PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS

Volume 4

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PERGAMON PRESS
NEW YORK · LONDON · PARIS
1957

CONTENTS

		Page
	PREFACE	vii
1	THE CONSTITUTION AND SYNTHESIS OF FATTY ACIDS *F. D. Gunstone, Department of Chemistry, The University, St. Andrews, Fife, Scotland*	1
2	THE NATURALLY OCCURRING ACETYLENIC ACIDS *E. M. Meade, Department of Food Chemistry, University of Toronto, Ontario, Canada*	45
3	THE SYNTHESIS OF GLYCERIDES *T. Malkin and T. H. Bevan, Department of Organic Chemistry, The University of Bristol, England*	63
4	LIPID WAXES *A. H. Warth, Wax Research Consultant, 29 York Court, Baltimore 18, Maryland, U.S.A.*	79
5	THE SYNTHESIS OF PHOSPHOLIPIDS *T. Malkin and T. H. Bevan, Department of Organic Chemistry, The University of Bristol, England*	97
6	THE LECITHINASES *D. J. Hanahan, Department of Biochemistry, University of Washington, Seattle, Washington, U.S.A.*	141
7	LIPID DYNAMICS IN ADIPOSE TISSUE *B. Shapiro, Department of Biochemistry, The Hebrew University—Hadassah, Medical School, Jerusalem, Israel*	177
8	DILATOMETRY *B. M. Craig, Associate Research Officer, Prairie Regional Laboratory, National Research Council, Saskatoon, Canada*	197
9	ULTRA-VIOLET SPECTROPHOTOMETRY OF FATTY ACIDS *G. A. J. Pitt and R. A. Morton, Department of Biochemistry, University of Liverpool, Liverpool, England*	227
	NAME INDEX	279
	SUBJECT INDEX	287

NAME INDEX

Abderhalden E. 126, 139
Abu-nasr A. M. 260, 264, 265, 267, 274
Adam, N. K. 81, 95
Adams, R. J. 16, 17, 40
Aggarwal, J. S. 230, 240, 258, 275, 278
Ahlers, N. H. E. 11, 39, 61, 239, 240, 241, 274
Ahmad, K. 34, 136, 42, 139
Allan, J. L. H. 61, 231, 232, 274
Allen, R. H. 120, 138
Allen, C. F. 231, 236, 242, 274
Allinger, N. L. 8, 39, 231, 274
Ames, D. E. 11, 21, 26, 27, 39, 41, 42
Anchel, M. 48, 51, 52, 53, 60, 61
Anderson W. E. 178, 180, 196
Anet, E. F. L. J. 55, 61
Anfinsen, C. B. 194, 195
Anker, H. S. 180, 182, 184, 194
Annau, E. 173, 175
Appleton, H. D. 156, 175
Armstrong, E. F. 4, 15, 38
Arnaud, A. 10, 39
Arnaud, M. 47, 48, 49, 61
Arnold, H. 102, 107, 116, 138
Ashton R. 19, 41
Astwood, E. B. 188, 194
Ault, W. C. 252, 274
Averill, H. P. 74, 77

Bachmann, G. B. 17, 41
Bachmann, W. E. 16, 40
Backderf, R. 254, 274
Badger, G. M. 25, 42
Baeder, D. H. 188, 196
Baer, E. 66, 70, 76, 77, 100, 102, 103, 104, 105, 108, 110, 111, 115, 116, 117, 119, 120, 124, 125, 126, 127, 128, 137, 138, 139, 149, 156, 175
Bagchi, P. N. 14, 40
Bailey, A. E. 198, 202, 203, 204, 205, 206, 208, 210, 211, 212, 215, 216, 219, 222, 223, 224, 225
Bailey, A. S. 231, 242, 274
Baldwin, A. R. 74, 77, 265, 268, 274
Baliga, M. N. 245, 263, 274
Balls, A. K. 162, 176
Ball, E. G. 172, 175
Baltes, J. 237, 239, 240, 241, 276
Banfi, D. 136, 139
Bannerot, R. A. 23, 41
Barbot, A. 15, 40
Barnes, R. H. 229, 274
Barrett, H. M. 187, 194
Barron, E. S. G. 181, 195
Barry, P. J. 72, 77
Baruch, H. 185, 195
Baruch, J. 5, 38
Bärwind, E. 66, 68, 76
Batik, J. 116, 138
Baudart, P. 8, 24, 26, 39, 41, 42
Bauer, S. T. 234, 235, 252, 277
Baur, F. J. 72, 77
Baylis, R. L. 107, 111, 113, 114, 115, 117, 119, 128, 138
Beadle, B. W. 247, 252, 254, 256, 274
Beckman, A. L. 243, 275
Begemann, P. H. 4, 7, 8, 15, 38
Behrens, M. 121, 138
Bell, S. H. 274
Bellucci, I. 66, 76
Benotti, J. 104, 138
Bentor, V. 189, 196
Bergemann, M. 65, 66, 67, 68, 76
Bergmann, W. 85, 245, 274
Bergström, S. 10, 16, 39, 40, 151, 175
Berk, L. C. 252, 270, 274
Bernhard, K. 179, 194
Bernheim, M. L. C. 173, 175
Bernheim, F. 173, 175
Bersin, T. 121, 138
Berthelot, M. 65, 76
Best, C. H. 187, 194
Bevan, T. H. 65, 70, 76, 77, 102, 104, 105, 107, 110, 111, 114, 115, 117, 119, 121, 127, 128
Beznak, A. B. L. 126, 139, 189, 194
Bharucha, K. E. 11, 39
Bhattacharya, R. 34, 42, 47, 61
Bidwell, E. 165, 176
Biehringer, J. 119, 138
Birch, A. J. 258, 268, 275
Black, H. K. 14, 37, 40, 50, 62, 236, 238, 242, 274
Black, H. C. 66, 67, 76
Bleyberg, W. 17, 40, 87, 95
Bloch, K. 182, 184, 195
Blomstrand, R. 150, 176
Bloor, W. R. 149, 176, 186, 195
Bodenstein, M. 59, 61
Boekenoogen, H. A. 4, 7, 8, 15, 38
Boelsing, F. 16, 17, 40
Böeseken, J. 8, 38
Bohlmann, F. 52, 54, 55, 57, 61
Boncoddo, N. F. 104, 121, 138
Boneard, N. 5, 38
Bongard, W. 264, 276
Boord, C. E. 12, 23, 39, 41
Booth, R. E. 244, 275
Boughton, B. W. 27, 42
Bounds, D. G. 17, 40, 61
Bowen, A. R. 15, 40
Bowman, R. E. 11, 21, 26, 27, 39, 41, 42
Boxer, G. E. 186, 196
Boxer, S. E. 17, 41
Boyd, H. M. 8, 11, 39, 239, 250, 252, 253, 254, 276
Boyle, E. 194, 195
Bradley, T. F. 249, 252, 270, 274
Braganca, B. M. 172, 175
Braude, E. A. 228, 231, 274

Brett, R. A. 11, 39, 239, 241, 274
Bretz 52, 61
Breusch, F. L. 17, 41
Brewster, L. E. 148, 164, 176
Brice, B. A. 235, 239, 240, 248, 249, 250, 251, 252, 253, 255, 256, 257, 264, 265, 268, 269, 274, 278
Brobeck, J. R. 181, 196
Brockhausen, R. 239, 240, 241, 276
Brode, W. R. 7, 11, 13, 38, 39, 40, 241, 247, 256, 277
Brodie, B. 156, 175
Brown, A. C. 28, 42
Brown, D. A. 65, 76, 117, 119, 120, 139
Brown, J. B. 7, 11, 13, 34, 38, 39, 40, 43, 82, 83, 85, 90, 93, 95, 96, 241, 247, 254, 256, 274, 277
Brown, R. K. 194, 195
Bruckner, V. 129, 139
Brunstrom, L. C. 88, 96
Bruun, T. 61
Bublitz, H. R. 194, 195
Buchnea, D. 105, 137
Buffington, R. M. 200, 225
Burch, W. J. N. 119, 125, 126, 138, 139
Burlew, J. S. 200, 202, 225
Bumpus, F. M. 34, 42
Büntgen, C. 25, 42
Burdon, K. L. 54, 61
Burr, G. O. 11, 13, 39, 229, 244, 246, 248, 252, 259, 260, 261, 267, 268, 270, 274, 275, 276, 277
Buu-hoï, N. P. 14, 40

Campbell, J. 187, 194, 195
Caravella, M. 234, 235, 252, 277
Carmichael, M. 14, 40
Carter, N. M. 66, 67, 68, 74, 76, 77, 121, 128, 130, 137, 138, 139
Carter, H. E. 143, 175
Cason, J. 8, 22, 39, 41, 229, 231, 239, 242, 274
Castille, A. 49, 50, 61, 236, 274
Cawley, J. D. 231, 252, 258, 274, 277
Celmer, H. 143, 175
Celmer, W. D. 35, 43, 54, 61
Chaikoff, I. L. 159, 175, 183, 185, 195
Chain, E. 126, 139
Champougny, J. 180, 195
Channon, H. J. 116, 138, 159, 175
Chargaff, E. 147, 175
Chechak, A. J. 231, 277
Cheftel, R. I. 243, 271, 276
Chegyi, G. 129, 139
Chen, C. 74, 77
Cherbuliez, E. 119, 126, 138
Cherkes, A. 194, 195
Chernick, S. S. 183, 185, 195
Chernovarova, A. A. 10, 39
Chibnall, A. C. 7, 38, 116, 138, 159, 175
Chimenes, H. 185, 195
Christenson, R. M. 22, 41
Christensen, P. K. 37, 43
Christensen, H. N. 114, 119, 126, 138
Chowers, I. 189, 190, 191, 193, 196
Chu, H. P. 163, 175
Chuit ,P. 16, 17, 40
Clarkson, C. E. 74, 77
Clement, G. 178, 181, 185, 189, 195
Clemo, G. R. 10, 39
Coenen, M. 25, 42
Cohen, N. H. 86, 96
Cohen, W. D. 72, 74, 77
Cohen, S. S. 147, 175
Collins, G. J. E. 81, 95
Collins, F. J. E. 6, 17, 38
Collins, F. I. 252, 274
Constantin, M. J. 147, 175
Contardi, A. 142, 144, 153, 167, 175
Conroy, E. A. 230, 239, 275
Cook, C. L. J. 62
Coops, J. 14, 40
Cornwell, D. G. 254, 274
Cowdrey G. W. 76
Craig, B. M. 72, 77, 203, 204, 205, 206, 208, 211, 212, 214, 223, 224, 226
Crombie, L. 8, 11, 17, 34, 35, 39, 40, 50, 61, 231, 239, 240, 241, 275
Cross, C. K. 7, 38, 244, 276
Crossley, A. 231, 242, 248, 258, 275
Crowe, R. W. 208, 226
Crum Brown, A. 14, 40
Cruz, A. O. 19, 41
Cullumbine, H. 189, 195
Czerny, W. 8, 38

Damaré, H. G. 257, 273, 277
Dann, W. J. 244, 275
Dauben, W. G. 16, 40
Daubert, B. F. 7, 38, 67, 68, 71, 72, 74, 77, 244, 259, 265, 266, 268, 274, 275, 277
Davenport, J. B. 258, 268, 275
Davidson, F. M. 148, 161, 175
Davies, R. H. 16, 17, 34, 36, 40, 41, 43
Davis, S. B. 230, 239, 275
De Groot, M. S. 16, 40
De Roy van Zuydewjin, E. 68, 72, 76
De, S. S. 152, 175
De Quant, J. C. 68, 72, 76
Debuch, H. 99, 113, 137
Delaby, R. 23, 41
Delange, R. 34, 42
Delcourt, Y. 81, 95
Dennison, A. C. 240, 258, 274
Desnuelle, P. 147, 175
Devine, J. 231, 242, 275
Diaper, D. G. M. 19, 41
Dickey, F. H. 132, 139
Dierichs, W. 25, 41, 42
Dollear, F. G. 204, 217, 225
Donahue, E. T. 90, 96
Donaldson, J. 241, 253, 262, 275
Dorinson, A. 87, 95
Drake, N. L. 22, 41
Dreike, A. 245, 264, 276
Drury, D. R. 181, 195
Dutka, F. 133, 139
Dutta, J. 10, 39
Dyer, J. W. W. 81, 95
Dykstra, H. B. 23, 41

Eckert, A. 10, 39

Eckey, E. W. 72, 77
Eddinger, C. C. 231, 277
Edisbury, J. R. 244, 246 275
Edwards, S. W. 172, 175
Eger, W. 178, 195
Egerton M. J. 122, 130, 132, 139
Eglinton, G. 16, 17, 33, 40, 42, 53, 62
Eisner, A. 90, 96
Eisner, U. 232, 275
Elieson 52, 61
Elliot, K. A. C. 173, 175
Elvidge, J. A. 232, 275
Eperjessy, A. 173, 175
Ercoli, A. 142, 144, 153, 167, 175
Evans, E. A. 231, 274
Evans, J. D. 270, 275

Fairbairn, 144, 145, 147, 153, 156, 157, 158, 167, 168, 175
Fairbourne, A. 65, 76
Fairweather, D. A. 14, 40
Farber, R. K. 187, 188, 195
Farmer, E. H. 244, 275
Faure, M. 99, 137
Faure, P. K. 230, 275
Favarger, P. 182, 183, 185, 192, 195, 196
Fawcett, D. W. 180, 195
Fazakerley, S. 231, 275
Feller, D. D. 182, 183, 195
Felszeghy, O. 173, 175
Felts, J. M. 183, 195
Ferguson, L. N. 228, 275
Fermo, M. W. 72, 77
Feulgen, R. 121, 138
Feuge, R. O. 218, 226
Fieser, L. F. 22, 41
Fickett, W. 132, 139
Findley, T. W. 85, 90, 93, 95
Fischer, E. 64, 66, 67, 68, 69, 70, 71, 73, 74, 76, 77
Fisher, N. 129, 136, 139
Fitz, A. 15, 40
Fodor, G. 129, 133, 136, 139
Fokin, S. 8, 38
Folch, J. 154, 175
Fonken, G. J. 231, 239, 274
Forbes, W. C. 8. 38
Foster, G. E. 76
Fraenkel-Conrat, H. L. 152, 176
Francis, F. 6, 15, 17, 19, 38, 40, 41, 81, 87, 95
Freeman, A. F. 239, 277
Friedel, R. A. 61
Front, J. S. 265, 275
Fulton, N. D. 202, 224, 225
Fusari, S. A. 34, 43

Gadient, F. 135, 139
Gaiser, C. J. 68, 76
Garner, W. E. 204, 225
Geddes, W. F. 203, 206, 208, 211, 212, 214, 223, 224, 226
Gensler, W. J. 25, 36, 37, 41, 43
Gerlach, J. 182, 183, 185, 195
Ghosh, B. N. 152, 175
Gianoli, G. 66, 76
Gibbs, R. H. 270, 278
Gibson, R. E. 200, 225
Gillam, A. E. 228, 244, 275
Ginsberg, H. F. 26, 42
Girvin, M. D. 24, 41
Glaser, C. 32, 42
Glick, F. J. 128, 139
Golding, J. 244, 275
Goldrine, L. S. 243, 275
Goldstein, M. S. 188, 196
Goljmov, V. P. 47, 62
Gordon, R. S. 194, 165
Grail, C. F. 182, 184, 196
Green, T. G. 90, 95
Greenberg, S. I. 267, 276
Greenfield, H. G. 59, 61
Greenlee, K. W. 12, 34, 39, 43
Gregory, G. I. 65, 67, 129, 130, 132, 134, 139
Grey, T. F. 26, 42
Griffiths, H. N. 11, 39
Grigor, J. 8, 39, 49, 61 238, 275
Grimme, C. 49, 61
Grob, C. A. 17, 41, 129, 132, 135, 139
Grollman, A. 34, 43
Gros, A. T. 204, 209, 210, 225
Grün, A. 8, 38, 65, 68, 69, 70, 74, 75, 76, 100, 101, 102, 109, 116, 126, 137, 138, 139
Guillot-Allègre, S. 23, 41
Gujot, O. 181, 189, 195
Gunstone, F. D. 4, 5, 7, 11, 38, 39, 49, 61, 232, 238, 239, 242, 274, 275
Gupta, R. S. 34, 43
Gupta, S. C. 240, 258, 275
Gupta, S. S. 240, 258, 275
Guth, F. 65, 69, 76

Hahn, P. F. 194, 195
Hahn, H. H. 229, 245, 261, 277
Hale, J. T. 65, 76
Hale, J. B. 17, 40
Halla, O. 10, 39
Hammond, E. G. 232, 241, 248, 251, 260, 261, 264, 265, 266, 267, 270, 271, 275
Hanahan, D. J. 144, 145, 147, 148, 149, 150, 151, 155, 156, 157, 159, 164, 171, 175
Hanks, D. P. 231, 242, 275
Hansen, C. 180, 195
Hansen, A. E. 270, 278
Hansson, G. 10, 39
Hare, G. H. 243, 275
Harrison, J. B. 130, 139
Harwood, H. T. 16, 40
Hasch, Z. 189, 194
Haskelburg, L. 10, 39
Hatt, H. H. 49, 61, 244, 263, 275
Haugaard, W. 182, 195
Hausberger, F. X. 181, 182, 183, 185, 189, 194, 195
Hausser, J. 16, 17, 40
Hawes, R. C. 243, 275
Hawthorne, N. J. 99, 137
Hayaishi, O. 144, 147, 152, 155, 156, 157, 175 176
Heilbron, I. M. 244, 276
Heinzelman, D. C. 234, 235, 239, 240, 252, 254, 258, 277
Helferich, B. 68, 76
Hellström, B. 54, 61
Hendrickson, J. 246, 276
Henriques, V. 180, 195
Herb, S. F. 13, 39, 232, 234, 241, 243, 245, 248, 250, 252, 253, 254, 257, 259, 260, 261, 262, 264, 265, 267, 270, 271, 272, 274, 175, 277

Herminghaus, H. 237, 276
Hersek, S. 17, 41
Hessel, L. W. 71, 77, 117, 138
Heyes, J. K. 263, 275
Hibbert, H. 66, 76, 121, 138
Hilditch, T. P. 4, 7, 11, 12, 13, 14, 15, 38, 39, 66, 67, 90, 96, 179, 180, 195, 230, 231, 242, 244, 245, 247, 248, 250, 251, 252, 253, 257, 258, 263, 265, 268, 274, 275
Hirai, H. 90, 96
Hirsch P. F. 185, 195
Hoevers, R. 8, 38
Hoff, M. C. 12, 39
Hofgaard, Kai. 202, 203, 208, 219, 220-221, 223, 224, 225
Hofmann, A. W. 14, 40
Hoffman, J. D. 208, 226
Hofmann, K. 4, 20, 38–41
Hogg, A. J. P. 8, 39, 238, 275
Holman, R. T. 231, 235, 242, 248, 252, 259, 260, 261, 263, 264, 265, 267, 270, 271, 274, 275, 276, 277
Holme, D. 62
Hook, W. E. 181, 195
Hopkins, C. Y. 126, 139
Horn, D. H. S. 84
Hougen, F. W. 84, 96
Howe, R. J. 73, 77, 107, 138
Howton, D. R. 16, 17, 34, 36, 40, 41, 43
Huber, W. F. 22, 34, 41
Hudak, E. S. 34, 43
Huisman, H. O. 231, 276
Humiston, C. G. 137, 139
Hundeshagen, F. J. 116, 138
Hunter, G. D. 8, 15, 39
Hunter, I. R. 72, 77, 110, 111, 138

Imhauser, K. 121, 138
Imhoffen, E. 52, 54, 61
Inukai, F. 126, 139
Irvine, J. C. 66, 76
Izar, A. 65, 76

Jachtorowisz, O. 182, 185, 195
Jacklin, A. G. 35, 43
Jackson, E. L. 126, 139
Jackson, F. L. 74, 77, 217, 226
Jackson, J. E. 8, 11, 39, 239, 250, 252, 253, 254, 276
Jacobs, T. L. 34, 42
Jacobson, M. 17, 41, 231, 275
Janicaud, J. 14, 40
Jeger, O. 85, 86, 96
Jelen, F. C. 200, 225
Jenny, E. F. 17, 41, 129, 132, 139
Johnson, A. W. 7, 10, 38, 39
Johnson, E. A. 54, 61
Johnson, J. R. 17, 34, 41, 42
Johnson, R. M. 270, 276
Jones, E. R. H. 33, 42, 48, 51, 52, 53, 61, 62, 231, 232, 246, 248, 274, 276
Jones, G. 200, 225
Jones, R. G. 22, 41
Jordan, L. A. 274
Jucker, O. 20, 41

Kabashima, A. 102, 107, 109, 110, 116, 138
Kade, F. 100, 116, 137
Kajanne, A. 248, 276
Kapp, R. 19, 41
Karrer, P. 16, 40
Kartha, A. R. S. 230, 276
Kass, J. P. 6, 11, 13, 38, 39, 232, 239, 240, 244, 246, 254, 268, 276, 277
Kates, M. 103, 104, 105, 108, 119, 124, 138, 149, 156, 160, 161, 162, 163, 165, 166, 175
Kaufmann, H. P. 237, 239, 240, 241, 276
Kavanagh, F. 60
Keller, M. 241, 276
Kendall, V. G. 35, 43
Kennedy, E. P. 170, 171, 176
Keppler, J. G. 4, 7, 8, 15, 38
Kester, E. B. 68, 72, 76, 77, 110, 111, 116, 138
Keston, A. S. 182, 195
Kielley, W. W. 172, 176
King, C. G. 67, 73, 74, 76, 77
King, A. M. 17, 19, 40, 41
Kirch, A. 70, 74, 76
Kiss, J. 129, 136, 139
Klein, P. D. 270, 276
Klenk, E. 5, 38, 99, 113, 137, 245, 264, 265, 276
Klevens, H. B. 229, 277
Knight, B. C. J. G. 163, 164, 176
Knoll, A. 19, 41
Koenig, H. 16, 40
Kolbe, H. 28, 42
Korn, E. D. 194, 195
Kornberg, A. 144, 147, 152, 155, 156, 157, 170, 171, 175, 176, 191, 195
Kon, G. A. R. 12, 39
Kon, S. K. 244, 275
Koonce, S. D. 82, 83, 95
Kraemer, E. A. 202, 205, 219, 222, 223, 224, 225
Kraft, F. 14, 15, 40, 65, 76
Kraybill, H. R. 12, 39, 246, 247, 248, 250, 251, 252, 254, 256, 265, 271, 274, 276
Kretchmer, N. 252 270, 274
Kreychunas, A. 22, 41
Kulkarni, B. S. 273, 277
Kummerow, F. A. 271, 277
Kuré, K. F. 189, 195
Kurth, E. F. 92, 95
Kutono, M. 83, 96
Kuwata, T. 83, 96

La Du, B. N. 156, 175
Landauer, S. R. 70, 77
Lane, J. F. 14, 40
Lange, W. 72, 77
Langheld, K. 126, 139
Lardelli, G. 86, 96
Latimer, W. M. 200, 225
Lazar, M. E. 68, 76
LeBreton, E. 153, 176, 180, 195
Lecomte, J. 23, 41
Lehmann, A. 10, 39, 232, 277
Lemon, H. W. 7, 15, 38
Lemmon, H. W. 244, 276
Leopold, W. 121, 138
Letch, R. A. 6, 38

Levene, P. A. 14, 15, 40, 81, 96, 128, 139, 147, 148, 149, 176
Levin, L. 187, 188, 195
Levy, B. B. 156, 175
Lewis, G. M. 164, 176
Lewis, J. F. 23, 41
Li, C. H. 152, 176
Libet, B. 173, 175
Liethelm, S. P. 49, 61, 237, 238, 242, 246, 276
Limpächer, R. 65, 76, 101, 109, 116, 126, 137, 138, 139
Lindlar, F. 33, 42, 264, 265, 276
Links, J. 16, 40
Linstead, R. P. 6, 12, 17, 38, 39, 40, 41, 60, 61, 232, 275
Lipmann, F. 148, 164, 176
Lipscomb, E. 259, 266, 277
Loeffler, O. H. 200, 225
Löfgren, N. 54, 61
Long, C. 147, 148, 165, 176
Long, C. N. H. 181, 196
Longenecker, H. E. 74, 77, 179, 195
Lovern, J. A. 244, 246, 275
Lucas, B. J. 187, 195
Lucas, H. J. 130, 139
Lucas, R. A. 4, 38
Luddy, F. E. 85, 96
Lumb, P. B. 34, 35, 43
Lumsden, J. S. 14, 40
Lundberg, W. O. 180, 195, 203, 204, 206, 208, 211, 212, 214, 215, 216, 223, 224, 226, 232, 241, 248, 251, 258, 260, 261, 264, 265, 266, 267, 269, 270, 271, 275
Lutton, E. S. 70, 71, 202, 217, 224, 225
Lycan, W. H. 17, 40
Lyman, C. P. 180, 195
Lythgoe, B. 55, 56, 61

Macinness, D. M. 8, 39
Maclennan, G. W. G. 12, 39
MacFarlane, M. G. 163, 164, 176
MacInnes, D. 238, 242, 275
MacKay, E. M. 181, 189, 195
Madhuranath, M. K. 238, 276
Maguire, M. F. 148, 164, 176
Mahadevan, V. 215, 216, 226
Makahara, W. 126, 139
Malet, G. 16, 17, 40
Maliphant, G. K. 117, 119, 120, 138
Malkin, T. 15, 17, 40, 65, 66, 67, 70, 71, 73, 74, 81, 99, 102, 104, 105, 107, 110, 111, 113, 114, 115, 117, 119, 120, 121, 122, 123, 127, 128, 129, 130, 131, 132, 134, 137, 138, 139
Manasse, M. 59, 61
Mangold, C. 8, 39
Manjunath, B. L. 238, 276
Mannhardt, H. J. 57, 61
Mansfield, G. H. 33, 42
Manzoor-i-khuda, M. 231, 275
Marble, A. 194, 196
Marchand, B. 121, 138
Markby, R. 61
Markley, K. S. 232, 276
Marsh, I. B. 182, 195
Martin, F. 104, 138
Martin, J. B. 64, 69, 76
Maruyama, T. 248, 276
Mason, R. G. 21, 41
Masoro, E. J. 183, 185, 195
Matthews, N. L. 11, 39
Mattson, S. 265, 276
Maurukas, E. 110, 111, 115, 128, 138
Maurukas, J. 104, 138
May, P. 239, 240, 274, 276
Maylott, A. O. 22, 41
McArthur, C. S. 126, 139
McDonald, J. 66, 76
McDowell, A. K. R. 252, 265, 276
McElroy, O. E. 74, 77
McGee, M. A. 61, 238, 242, 275
McGhie, J. F. 26, 42
McInnes, D. 61
McKinney, R. S. 234, 239, 240, 277
McLean, J. 8, 39, 61, 238, 242, 274, 275
McMeekin, T. L. 126, 139
McTaggart, N. E. 11, 39
McTaggart, N. G. 239, 241, 274
Mead, J. F. 245, 276
Meade, E. M. 50, 61, 236, 237, 238, 242, 274
Meader, A. L. 16, 40
Meakins, G. D. 57, 61
Meara, M. L. 71, 74, 77, 230, 275
Meerberg, W. 71, 74, 76, 77
Mehltretter, C. L. 148, 149, 176
Melamed, S. 22, 41
Mendel, H. 14, 40
Mendel, L. 178, 196
Menon, K. N. 230, 276
Meyer, J. D. 81, 96
Meyerhof, O. 172, 176
Mijovic, M. V. 85, 96
Mildner, P. 33, 42
Miles, A. A. 164, 176
Miles, E. M. 164, 176
Miller, E. S. 229, 246, 274, 276
Miller, F. A. 62
Miller, W. R. 20, 41
Milstein, S. M. 182, 183, 195
Mirski, A. 182, 195
Mitchell, J. H. 12, 39, 246, 247, 248, 250, 251, 252, 254, 265, 271
Mitter, P. C. 14, 40
Moldtmann, H. G. 121, 138
Moore, T. 13, 39, 244, 246, 275, 276
Morelec-Coulon, M. J. 99, 137
Moretti, J. 243, 271, 276
Morgan, G. T. 15, 40
Morton, I. D. 117, 138
Morton, R. A. 4, 12, 38–9, 244, 246, 247, 250, 251, 253, 257, 258, 265, 275, 276
Moureu, C. 34, 42
Mowry, D. T. 7, 13, 38, 40, 241, 247, 256, 277
Murray, K. E. 82, 83, 84, 87, 96
Murray, M. 218, 226
Muller, A. 208, 226
Müller, H. 58, 62
Myers, G. S. 11, 39

Nafziger, H. 121, 138
Narayan, K. A. 273, 277
Nath, B. 230, 278

Naudet, M. 147, 175
Newcombe, A. G. 105, 137
Newman, M. S. 34, 43
Nevenzel, J. C. 16, 40
Neville, H. A. 8, 38
Nichols, P. L. 13, 39, 232, 239, 250, 252, 253, 254, 257, 259, 264, 274, 277, 278
Niyogy, S. C. 34, 43
Noller, C. R. 23, 41
Norcia, L. N. 179, 195
Normann, W. 201, 225
Norris, W. P. 129, 137, 139
Nunn, W. 241, 278
Nunn, J. R. 4, 38
Nygaard, A. P. 172, 176

Obata, Y. 103, 138
O'Connell, P. W. 174, 176, 259, 266, 277
O'Connor, R. T. 218, 226, 234, 235, 239, 240, 243, 252, 254, 257, 258, 273, 277
Oestreicher, T. 182, 196
Okniaki 189, 195
Oleksyshyn, N. L. 270, 275
Olley, J. 170, 176
O'Neill, L. A. 240, 258, 274
Outhouse, E. L. 126, 139
Overbeck, O. 47, 61
Overley, C. A. 67, 76

Pääbo, K. 16, 40, 151, 175
Pack, F. C. 218, 226, 234, 239, 240, 254, 258, 277
Pantaleon, E. 153, 176
Papa, D. 26, 42
Paschke, R. F. 8, 11, 39, 234, 239, 250, 252, 253, 254, 263, 276, 277
Patel, C. B. 250, 251, 252, 253, 275
Pathak, S. P. 7, 38
Payne, R. W. 188, 195
Pedelty, W. H. 179, 195
Penny, I. F. 147, 148, 176
Perkins, G. A. 19, 41
Pfähler, E. 70, 76
Phillips, G. E. 129, 139
Pigulevskiĭ, G. 10, 39
Pihl, A. 182, 184, 195
Pillemer, L. 165, 176
Piper, S. H. 6, 7, 15, 17, 38, 40, 81, 82, 95
Planck, R. W. 234, 239, 240, 254, 258, 277
Platt, J. R. 229, 277
Plattner, Pl. A. 26, 42
Plimmer, R. H. A. 119, 125, 126, 128, 138, 139
Poffrath, H. 126, 139
Polatnick, J. 60
Polgar, N. 231, 242, 274
Polonovski, M. 178, 196
Polonovski, J. 178, 196
Poole, A. G. 99, 137
Popják, G. 8, 15, 39
Porck, A. J. E. 72, 77
Posternak, S. 10, 39
Pradhan, M. K. 26, 42
Pricer, W. E. 191, 195
Pricer, W. W. 170, 176
Priest, G. W. 8, 38
Privett, O. S. 258, 269, 277
Prŏstenik, M. 16, 40, 130, 134, 137, 139

Quagliariello, G. 180, 194, 196
Quastel, J. H. 172, 175
Quigley, T. W. 194, 195

Raben, M. S. 188, 194
Radell, J. 90, 96
Ralston, A. W. 16, 40, 81, 96
Raphael, R. A. 7, 29, 33, 34, 35, 36, 38, 42, 43, 47, 53, 59, 61
Rebello, D. 7, 38, 244, 277
Reed, L. 178, 196
Reid, E. E. 81, 96
Renold, A. E. 194, 196
Renshaw, R. R. 125, 126, 139
Reshef, L. 193, 196
Richardson, D. 249, 252, 270, 274
Richardson, G. H. 265, 272, 278
Ridout, Y. H. 187, 194
Rieckehoff, I. G. 259, 277
Riemenschneider, R. W. 13, 39, 232, 241, 243, 248, 250, 252, 253, 254, 257, 259, 260, 262, 263, 264, 265, 270, 271, 272, 275, 277
Rigg, J. G. 66, 76
Riley, J. P. 5, 7, 12, 38, 39, 50, 61, 250, 251, 252, 253, 257, 258, 264, 265, 274, 275
Rinehart, K. L. 231, 274
Rittenberg, D. 179, 180, 182, 186, 196
Roberts, R. L. 72, 77, 110, 111, 138
Robeson, C. D. 231, 277
Robinson, G. M. 17, 18, 19, 41
Robinson, R. 18, 19, 41, 231, 242, 274
Roche, J. N. 74, 77
Rockwell, H. E. 137, 139
Rodbell, M. 145, 149, 171, 175
Rodda, H. J. 25, 42
Rolf, I. P. 147, 149, 176
Romberg, P. 65, 76
Rose, G. 182, 183, 185, 190, 191, 193, 196
Rose, W. G. 71, 77, 102, 110, 111, 116, 138
Rosenberg, I. N. 188, 194, 196
Ross, H. K. 15, 40
Ross, W. A. 26, 42
Roth, D. A. 22, 41
Roth, F. B. 165, 176
Rottenberg, M. 16, 40
Rountree, J. M. 16, 40
Ruska, T. 182, 196
Rusoff, I. I. 229, 274, 277
Russell, M. 110, 111, 138
Russell, W. C. 7, 38, 232, 239, 275
Rutman, R. J. 182, 183, 195
Ruzicka, L. 26, 42, 85, 86, 96
Ryan, A. J. 258, 268, 275
Ryder, E. A. 204, 225
Rydon, H. N. 70, 77

Sabetay, S. 76
Sakuragi, T. 271, 277
Salcedo, J. 186, 196
Saletore, S. R. 34, 42, 47, 61
Sallay, L. 133, 136, 139
Sasse, W. H. F. 25, 42
Saunders, J. B. 200, 225
Sax, S. M. 4, 38
Scanlon, J. T. 85, 96
Schacht, P. 69, 76
Schellenberg, H. 86, 96

Schmid, H. 10, 39, 232, 277
Schmidt, G. 121, 125, 138
Schoelly, D. 192, 196
Schoenfeld R. 82, 83, 84, 87, 96
Schoenheimer, R. 179, 180, 182, 186, 196
Schon, L. 81, 96
Schormüller, A. 128, 139
Schuette, H. A. 22, 41, 65, 76
Schwamberger, E. 14, 40
Schwartz, A. M. 34, 42
Schwartz, H. M. 237, 242, 246, 276
Schweizer, A. 17, 41
Schwenk, E. 26, 42
Scoz, G. 194, 196
Sedgwick, V. E. 252, 274
Segal, K. 135, 139
Seher, A. 50, 61, 236, 242
Seifter, J. 188, 196
Senti, F. R. 239, 278
Shaeffer, B. B. 252, 274
Shaeffer, G. 189, 195
Shafrir, E. 193, 196
Shakespeare, N. E. 230, 239, 275
Shapiro, B. 153, 154, 157, 167, 178, 180, 182, 183, 185, 189, 190–191, 193, 196
Shapiro, D. 130, 135, 139
Shaw, B. L. 33, 42
Sherwood, F. W. 180, 196
Shimo-oka T. 245, 263, 264, 278
Shirley, D. A. 22, 41
Shorland, F. B. 179, 196, 263, 275
Shriner, R. L. 17, 41
Shrivastava, R. K. 268, 275
Shurbagy, M. R. 71, 77
Sickel, H. 126, 139
Sidhu, S. S. 74, 77
Sieber, H. 68, 76
Silk, M. H. 55, 61, 229, 245, 262, 277
Simonova, N. 10, 39
Simonsen, J. L. 34, 42, 47, 61
Sinclair, R. G. 149, 176, 186, 196
Singleton, W. S. 204, 205, 206, 209, 210, 211, 215, 216, 217, 218, 222, 225
Skraup, S. 14, 40
Slaton, W. H. 245, 276
Slotta, K. H. 152, 176
Smernov, V. S. 47, 62
Smit, A. 231, 276
Smit, W. C. 8, 39
Smith, D. B. 70, 76, 77, 114, 138
Smith, F. H. 180, 196
Smith, J. C. 16, 19, 34, 35, 40, 41, 43, 230, 275
Smyth, C. P. 208, 226
Snider, R. H. 149, 176
Solomons, I. A. 35, 43, 54, 61
Sondheimer, F. 34, 35, 36, 42, 43
Sorensen, N. A. 37, 43, 48, 51, 52, 54, 55, 57, 62
Soutar, C. W. 66, 76
Sowden, J. C. 73, 77
Stainsby, W. 90, 96
Ställberg-Stenhagen, S. 20, 41
Stanacev, N. 130, 134, 139
Stancer, H. C. 125, 127, 139
Stansbury, M. F. 257, 258, 273, 277
Stark, S. M. 257, 273, 277
Steele, J. M. 156, 175
Steger, A. 48, 49, 62
Stegerhoek, L. J. 118, 138
Steinhauser, H. 179, 194
Stenhagen, E. 3, 20, 21, 38, 41
Step, W. 121, 138
Stern, E. S. 228, 275
Stern, I. 182, 183, 185, 189, 190, 196
Stern, M. H. 231, 277
Sternberg, H. W. 61
Stetten, D. 182, 184, 186, 187, 196
Stetter, H. 25, 41, 42
Stevens, R. 10, 39
Stickney, M. E. 243, 275
Stimmel, B. F. 67, 76
Stotz, E. 174, 176
Strong, F. M. 34, 42, 43
Struve, W. S. 16, 40
Stubbs 263
Sumner, J. B. 172, 176
Sumrell, G. 8, 39, 229, 231, 242
Sunko, D. E. 137, 139
Sutton, D. A. 10, 39, 229, 245, 263, 264, 275
Suzuki, B. 248, 276
Swain, M. L. 235, 239, 240, 248, 249, 250, 251, 252, 255, 256, 257, 264, 265, 268, 269, 274, 278
Swift, A. N. 245, 274
Szmuszkovicz, J. 22, 41
Szumer, A. Z. 61, 244, 263, 275

Tausig, F. 20, 41
Tayler, J. L. 239, 240, 241, 275
Taylor, F. A. 15, 40
Taylor, P. B. 22, 41
Taylor, J. L. 11, 39
Taylor, W. R. 34, 43
Teeter, A. J. 10, 39
Tepperman, J. 181, 196
Thannhauser, S. J. 104, 121, 138
Thomas, G. R. 25, 36, 37, 41, 43
Thompson, A. 244, 276
Thoshin, F. 189, 195
Tiedt, J. 83, 96
Tietz, A. 191, 196
Todd, Sir A. R. 117, 138
Tolberg, W. 8, 11, 39, 239, 250, 252, 253, 254, 276
Tomecko, C. G. 16, 40
Tonks, D. B. 120, 138
Tookey, H. L. 162, 164, 176
Tool, H. Q. 200, 225
Tove, S. B. 180, 196
Toyama, Y. 24, 41, 90, 96, 245, 263, 264, 278
Trippett, S. 55, 61
Truter, E. V. 83, 96
Tsuchiya, T. 245, 278
Tuerkischer, E. 181, 182, 196
Tullis R. 17, 41
Turner (Jr.), A. 85, 96
Turner, L. D. 145, 149, 175

Uhlenbroek, J. H. 116, 117, 118, 138
Ulrich, H. 17, 40, 87, 95
Ulzer, F. 116, 138
Utzinger, H. 129, 139
Uziel, M. 153, 155, 156, 157, 167, 169, 176

Vaeck, S. V. 203, 204, 208, 219, 226

Vandenheuvel, F. A. 16, 40, 265, 272, 278
Vanin, I. I. 10, 39
Varma, J. P. 230, 278
Varrentrap, F. 12, 15, 39
Van Heyningen, W. E. 165, 176
Van Leeuwen, P. H. 231, 276
Van Lohuisen, O. E. 71, 77
Van Loon, J. 17, 40, 48, 49, 62
Van Rij, J. H. 231, 276
Van Roon, J. D. 67, 76, 121, 139
Van Slyke, D. D. 154, 175
Van der Hulst, L. J. N. 239, 278
Van der Lee, J. 68, 71, 72, 74, 76, 77
Van der Linden, D. 17, 40
Van der Neut, J. H. 118, 138
Vercamer, R. 164, 175
Verkade, P. E. 67, 68, 71, 72, 74, 75, 76, 77, 116, 117, 118, 121, 138
Velick, S. F. 88, 96
Vernon, A. A. 15, 40
Vicknair, E. J. 209, 217, 218, 226
Voit, K. 121, 138
Volbert, F. 239, 240, 241, 276
von Holdt, M. M. 237, 242, 246, 276
von Mikusch, J. D. 3, 13, 38, 39, 40, 252, 276
Voser, W. 85, 96
Vroege, A. K. 72, 74, 77

Wagner-Jauregg, T. 116, 119, 138
Walborsky, H. M. 36, 43
Waldron, 270, 275
Walker, J. 14, 28, 40, 42
Wallis, E. S. 14, 40
Ward, T. L. 204, 217, 225, 241, 278
Ware, J. C. 125, 139
Webley, D. J. 123, 139
Weedon, B. C. L. 14, 17, 28, 33, 37, 40, 42, 50, 61, 62, 236, 238, 242, 274
Weenink, R. O. 245, 278
Weil, R. 187, 196
Weisler, L. 231, 277
Weiss, S. B. 171, 176
Weissmann, D. 189, 196
Weitkamp, A. W. 83, 88, 96
Wells, H. G. 178, 196
Weniger, H. 119, 126, 138
Wender, I. 61
Wertheimer, E. 178, 180, 181, 182, 189, 196
West, C. J. 14, 40
Westermeyer-V. W. 188, 194
Wheeler, D. H. 4, 8, 11, 38, 39, 234, 239, 250, 252, 253, 254, 263, 276, 277
Whitcutt, J. M. 229, 245, 264, 278
Whitham, G. H. 246, 248, 276
Whiting, M. C. 16, 17, 33, 40, 42, 53, 57, 62, 231, 232, 246, 248, 274, 276
Widmer, W. 26, 42
Wiese, H. F. 270, 278
Wildemuth, A. 119, 138
Wilds, A. L. 16, 40
Wiljams, W. W. 47, 62
Wilke, G. 58, 61
Wille, R. L. 202, 224, 225
Williams, D. E. 22, 41, 231, 274
Williams, E. F. 7, 38
Willis, J. A. V. 17, 19, 40, 41
Wilson, B. R. 74, 77
Wilson, C. L. 254, 274
Winter, G. 241, 278
Wirz, W. 86, 96
Witham, G. H. 33, 42
Witka, F. 69, 76
Witnauer, L. P. 239, 260, 275, 278
Wittenberg, J. 171, 176
Wohl, H. 68, 70, 74, 76
Woltemate, M. L. 7, 38
Wool, I. G. 188, 196
Wotiz, J. H. 34, 43, 57, 61, 62

Yamamoto, T. 24, 41
Yarboro, C. L. 180, 196
Yates, P. 16, 40
Yosii, S. 180, 196
Yosinaga, T. 159, 176
Young, A. C. 20, 41
Young, C. T. 180, 196
Young, H. H. 66, 76

Zaky, Y. A. H. 230, 275
Zamecnik, P. G. 148, 176
Zscheile, F. P. 12, 39, 246, 247, 248, 250, 251, 252, 254, 271, 276

SUBJECT INDEX

ACETYLENIC ACIDS, history of 47
Acetylenic acids, naturally occurring
chemical properties 57
infra-red spectra 57
isolation of 56
physiological properties 59
properties used in separation 56
sources of 48
synthesis 59
ultra-violet spectra 57
Acetylenic glycerides, sources of 48
Adipose tissue
factors determining composition 178
effect of diet 179
effect of localization and temperature 180
fatty acid synthesis in 181
lipid dynamics in 178

DEPOT FAT, mobilization and deposition of 186
humoral regulation 187
in vitro systems 189
methods of investigation 186
nervous regulation 189
Dilatometers 203
"apparent contractions" in 203
confining liquid 203
construction and operation of 200
types of 201
types of bulb 202
Dilatometry
conclusions on use of 225
errors in 200
of fatty acids 204
of methyl esters of fatty acids 205
of mono- and di-glycerides 209
of synthetic glyceride mixtures 212
pretreatment of samples 204
use in determination of solids content of fats and oils 223
use in study of natural products 219
use in study of polymorphism 215
of acetostearins 218
of cocoa butter 219
of monostearin 217
of stearic acid 217
of tristearin 215
of tung oil 218

FATTY ACIDS
anodic synthesis
Fatty acids (*contd.*)—
constitution and synthesis 3
determination of constitution 3
position of hydroxy and keto groups 5
use of hydrogenation 4
use of oxidation 4
preparation by chain degradation 13
by loss of more than one carbon atom 14
by loss of one carbon atom 14
preparation by chain extension 15
by addition of one carbon atom 15
by addition of two carbon atoms 16
preparation by condensations 18
use of ethyl acetoacetate 18
use of malonic ester 21
use of organo-metallic compounds 22
preparation by methods involving no change in chain length 6, 7
hydrogenation 6
oxidation 7
preparation 6
from closely related compounds 6
preparation using cyclic compounds as chain extenders 25
synthesis
by use of acetylene intermediates 29, 32, 33, 37, 38
miscellaneous methods 38
unsaturated, preparation of 7
by condensations 26
by dehalogenation 10
by dehydration of hydroxy compounds 7
by dehydrohalogenation 8
by elaidinization 11
by migration of unsaturated centres 12

GLYCERIDES, synthesis of 64
1:2-diglycerides 72
1:3-diglycerides 69
mixed diglycerides 73
monoglycerides 65
triglycerides 74
diacid 74
monoacid 74
triacid 74

Glyceride acetylenes
bolekic acid 50
isanic or erythrogenic acid 49
isanolic acid 50
tariric acid 48
ximenynic or santalbic acid 49
ximenynolic acid 49

Hydrolytic enzymes 144

Lecithin metabolism, possible enzymes involved in 165
hydrolytic type 166
isomerase 167
oxidation-reduction 171
synthetic 169
Lecithinase A 144
general application to biochemical problems 149
general characteristics and typical reaction systems 144
purification and properties 151
substrate specificity and site of action 147
Lecithinase B 152
general remarks on reaction of 153
preparation 157
reaction systems and substrate requirements 153
Lecithinase C 158
activation of enzyme and substrate requirements 162
primary mode of attack 159
Lecithinase D
general considerations 163
purification procedures 165
Lecithinases 142
nomenclature 142
Lipid waxes 80
alcohols of 80
amyrins and lupeol 86
aromatic acids of 92
branched alcohols from, melting points of 84
determination of triterpene alcohols 85
dihydric alcohols, melting points of 84
esters of 93
fatty acids of 87
hydroxy acids 89, 90
iso and ante-iso acids 88
physical properties of 87
separation of *n* acids by methylation 87
iso and ante-iso alcohols 83
lactones and lactides 83
δ lactones in wool wax 92
occurrence of wax alcohols in nature 82

Lipid waxes, (*contd.*)—
physical properties of *n* alcohols 81
densities 81
melting and boiling points 81
X-ray spacings 81
separation of *n* alcohols 82
amplified distillation technique 82
fractional distillation of acetylated alcohols 82

Non-glyceride acetylenes 51
the C_8 group
agrocybin 51
clitocybin 51
diatetryne 51
The C_{10} group 51
dehydromatricaria ester 52
dihydromatricaria ester 51
isano decenediyne 51
lachnophyllum ester 51
matricaria ester 52
matricarianol 52
the C_{11} group 52
nemotin 53
nemotinic acid 52
odyssin 53
the C_{12} group 54
artemisia ketone 54
centaur X 54
the C_{13} group 54
*iso*mycomycin 54
mycomycin 54
the coreopsis poly-ynes 54
trienetriynyl acetate from *Carlina vulgaris* 54
the C_{14} group 55
anacycline 55
anacycline conjugated enediyne 55
artemisia ketone 55
the C_{17} group 55
cicutol 56
cicutoxine 56
oenanotoxine 55
oneanthetol 55
oenanthetone 55

Phosphatidic acids, synthesis of 116
1- and 2-acyl batyl phosphates 118
bis-glycol phosphatidic acids 120
bisphosphatidic acids 119
dialkylhydrogen phosphates 120
glycol analogues of phosphatidic acids 118
higher alkyl dihydrogen phosphates 119

Phospholipids
miscellaneous preparations 124
choline phosphate 125
ethanolamine phosphate 126
L-α-glyceryl phosphoryl choline 124
O-(*L*-α-glyceryl phosphoryl) ethanolamine 125
serine-3-phosphate 128
silver salt of phenyl phosphoryl-N-carbobenzoxy ethanolamine 128
synthesis of 98
alkyl β-aminoethyl phosphates 114
cephalin (phosphatidyl ethanolamine) 109
chimyl and batyl analogues of cephalin 113
glycol cephalins 114
glycol lecithins 108
lecithin (phosphatidyl choline) 100
lyso cephalin 111
lyso lecithin 107
phosphatidyl serine 115
Plasmalogens, synthesis of 121

Sphingolipids 128
synthesis of 128
dihydrosphingosine 129, 130, 131, 132, 133
sphingine 137
sphingosine 134, 135, 136

Ultra-violet absorption spectra 234
absorbing impurities in fatty acids 234
accuracy of spectroscopic analysis 238
acetylenic acids 235
alkaline isomerization of unconjugated fatty acids 243
adaptation to semi-micro and micro scale 270
artifacts 268
calibration by experiments on pure acids 252
concentration of alkali 258
correction procedures 254
development of analytical method 246
efficacy of method 272
solvent for isomerization 251
standard methods 271
temperature 251
time of heating 248
of naturally occurring acids 228

CONTENTS OF PREVIOUS VOLUMES

Volume 1

The Molecular Structure and Polymorphism of Fatty Acids and their Derivatives. T. Malkin

Sterols. Werner Bergmann

Structure and Properties of Phosphatides. P. Desnuelle

Chromatography of Fatty Acids and Related Substances. R. T. Holman

Derivatives of the Fatty Acids. H. J. Harwood

Volume 2

The Polymorphism of Glycerides. T. Malkin

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Some Aspects of the Intestinal Absorption of Fats. Sune Bergström and Bengt Borgström

Metabolism of the Steroid Hormones. Leo T. Samuels

PREFACE

THE level of development of a field of science can only be as high as its methodology will allow. The rapid development of physical methods of analysis of lipid substances and the consequent flood of information obtained has sharpened the interest in the chemistry and biochemistry of lipids. This series of volumes is intended to group together authoritative treatments of subjects of current interest to chemists in the field of lipids. Because of the heterogeneity of the lipids themselves and the variety of techniques now being used, the individual volumes are necessarily non-uniform. It is the hope of the editors that the series will provide a convenient and comprehensive source of information to workers in the field of lipids.

The technology of fats will be included in this series, but will be treated in separate volumes, the first of which will be Volume 5.

THE EDITORS

1

THE CONSTITUTION AND SYNTHESIS OF FATTY ACIDS

F. D. Gunstone

CONTENTS

		Page
I.	INTRODUCTION	3
II.	THE DETERMINATION OF THE CONSTITUTION OF A FATTY ACID.	3
	A. Hydrogenation	4
	B. Oxidation	4
	C. Hydroxy and Keto Acids	5
III.	THE PREPARATION OF FATTY ACIDS FROM CLOSELY RELATED COMPOUNDS.	6
	A. Methods involving no change of chain length	6
	1. Hydrogenation of unsaturated acids	6
	2. Oxidation of alcohols or aldehydes	7
	3. Preparation of unsaturated acids by methods resulting in an increase in the amount of unsaturation	7
	(*a*) Dehydration of hydroxy compounds	7
	(*b*) Dehydrohalogenation of halogeno compounds	8
	(*c*) Dehalogenation of vicinal dihalides	10
	4. Preparation of unsaturated acids by methods occurring without change in the amount of unsaturation	11
	(*a*) Change of configuration (elaidinization)	11
	(*b*) Migration of unsaturated centres	12
	B. Methods involving chain degradation	13
	1. Reactions resulting in the loss of one carbon atom	14
	2. Reactions resulting in the loss of more than one carbon atom	14
	C. Methods involving chain extension by one or two carbon atoms	15
	1. Chain extension by one carbon atom	15
	2. Chain extension by two carbon atoms.	16
IV.	THE PREPARATION OF FATTY ACIDS BY UNION OF SMALLER MOLECULES	18
	A. Condensations involving the use of ethyl acetoacetate or malonic ester as a coupling unit.	18
	1. Methods using ethyl acetoacetate	18
	2. Methods using malonic ester	21
	B. Condensations using organometallic compounds	22
	C. The use of cyclic compounds as chain extenders	25
	D. Condensations yielding acyloins or alkoxyketones and thence unsaturated acids	26

Contents

E. Anodic syntheses 28
F. The preparation and use of acetylenic intermediates 29
1. Condensation reactions of acetylenic compounds 29
2. The conversion of acetylenic intermediates to aliphatic acids . 32
3. Syntheses involving the use of organometallic acetylenic compounds 33
4. Syntheses involving oxidative coupling 37
G. Other methods 38

REFERENCES 38

1

THE CONSTITUTION AND SYNTHESIS OF FATTY ACIDS

F. D. Gunstone

I. Introduction

Although this review is concerned with methods of determining the structure and of synthesizing straight-chain fatty acids, the greater part is given to discussion of the methods of synthesis, since many important advances in this field have been reported within the last decade. The branched-chain compounds are the subject of a separate chapter in this series.[1]

The discussion on synthetic methods is conveniently divided into two parts; one part treats those methods which result in the modification of some closely related compound, and the other part is concerned with condensation reactions by which long-chain acids may be produced. It is in this latter section that the greatest advances have been made, resulting in methods of preparing many complex unsaturated acids. These more modern approaches are marked by close stereochemical control over configuration resulting from a fuller knowledge of stereospecific reactions. The application of this knowledge could lead to a greater and more productive use of closely related compounds in the synthesis of unsaturated long-chain acids, and there is some evidence that this is now being appreciated.

Emphasis is here placed on methods rather than on products, though several examples of each method are described and references given to further applications. The examples quoted are mainly those leading to naturally occurring acids or to closely related compounds. Since classification is according to method rather than product, saturated and unsaturated acids are sometimes grouped together, though where a method leads to acids of both types an attempt has been made to deal with one group first.

This chapter is not meant to be a comprehensive account of the work done in this field, but a critical discussion of the methods of synthesizing straight-chain compounds.

II. The Determination of the Constitution of a Fatty Acid

With the methods now available, determining the structure of an unbranched fatty acid is not the difficult problem it once was, indeed the main difficulty is usually the isolation of the acid in a state sufficiently pure for further investigation, a problem which is outside the scope of this chapter.

Having isolated the acid it is frequently sufficient to investigate the products of hydrogenation and oxidation and the ultra-violet and infra-red spectra. Study of the spectra assists in determining the relative positions of the unsaturated centres and the configuration about ethylene bonds. The use of ultra-violet and infra-red spectra has been fully discussed in this series[2, 3] and no further reference is made to them except to note that their importance cannot be over-emphasized.

A. Hydrogenation

Hydrogenation is effected catalytically, nickel, platinum, or palladium being used as catalyst. If the reaction is carried out quantitatively it is possible to estimate the extent of unsaturation which may then be usefully compared with the iodine value. When the iodine value is lower than that expected from hydrogenation this may indicate the presence of conjugated unsaturation, of double bonds close to the carboxyl group, of acetylenic compounds, or of other reducible (e.g. keto) groups. If the iodine value exceeds that indicated by hydrogenation this may be due to the presence of hydroxyl groups which sometimes interact with Wijs reagent.[4]

The product of hydrogenation is generally an *n*-saturated acid, less frequently it is a hydroxy acid resulting from a keto, epoxy, or hydroxy acid, or an alkyl substituted acid indicating the presence of an alkyl substituent or a cyclic system.[5, 6] The saturated acid is identified by elementary analysis, by its neutralization equivalent, melting point, or crystal spacings measured by X-rays, and by the preparation of appropriate derivatives. The very similar properties of the higher members of this series sometimes make their purification and identification difficult. It is then that the measurement of crystal spacings becomes particularly useful.

B. Oxidation

A major problem in assigning a structure to an unknown acid involves the position of the unsaturated centres and this may sometimes be solved by identification of the fission products resulting on oxidation. It is important that the products be obtained in high yield and purity and for this purpose oxidative cleavage is usually effected by potassium permanganate in acetone or acetic acid solution, or by ozonolysis.

Permanganate oxidation has been frequently used in structure studies and the claim[7] that the products are obtained in high yield and free of all but very minor amounts of further degradation products has recently been confirmed (1950).[8] The method is equally applicable to mono-, di-, and tri-ethenoid and to acetylenic acids, and the products after subsequent hydrolysis (a monobasic and one or more dibasic acids) are generally separable by steam distillation, monobasic acids up to C_{10} being conveniently volatile. The structure of the unsaturated compound follows from the composition of the oxidation products as illustrated in the following examples:

$$CH_3\cdot[CH_2]_5\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2Me \xrightarrow[\text{(ii) Hydrolysis}]{\text{(i) Oxidation}} CH_3\cdot[CH_2]_5\cdot CO_2H + HO_2C\cdot[CH_2]_7\cdot CO_2H$$

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2Me \rightarrow CH_3\cdot[CH_2]_4\cdot CO_2H + HO_2C\cdot CO_2H^{*} + HO_2C\cdot[CH_2]_7\cdot CO_2H$$

$$CH_3\cdot[CH_2]_3\cdot CH{:}CH\cdot CH{:}CH\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2Me \rightarrow CH_3\cdot[CH_2]_3\cdot CO_2H + HO_2C\cdot CO_2H + HO_2C\cdot[CH_2]_7\cdot CO_2H.$$

The structure of many unsaturated acids has been determined or confirmed by identification of the products of ozonolysis. These are either the same acids as those resulting from permanganate oxidation or the corresponding aldehydes. Decomposition of a mono-ozonide with water gives four products, but only the two aldehydic products result under reducing conditions, and only the two acidic products with hydrogen peroxide.

$$CH_3\cdot[CH_2]_7\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H \xrightarrow[\text{(ii) } H_2O]{\text{(i) } O_3} \begin{cases} CH_3\cdot[CH_2]_7\cdot CHO \\ \qquad + OHC\cdot[CH_2]_7\cdot CO_2H \\ CH_3\cdot[CH_2]_7\cdot CO_2H \\ \qquad + HO_2C\cdot[CH_2]_7\cdot CO_2H \end{cases}$$

Klenk and Bongard[8a] have extended this procedure to the study of polyethenoid acids.

C. Hydroxy and keto acids

The position of hydroxyl or keto groups in long-chain acids is readily determined by application of Baruch's procedure.[9] Although this method does not give wholly unambiguous results when applied to unsaturated acids,[10] it can be satisfactorily used for hydroxy or keto acids. The method is illustrated for the hydroxy acid present in isano oil.[11] A concentrate of the unsaturated hydroxy acid was esterified, hydrogenated, and oxidized to a methyl keto-stearate. The syn and anti oximes of this compound give amides when submitted to Beckmann rearrangement, and these on hydrolysis each afford two products. The identification of these clearly defines the position of the keto or hydroxyl group originally present.

$$CH_3\cdot[CH_2]_9\cdot CO\cdot[CH_2]_6\cdot CO_2Me$$

$$\downarrow$$

$$CH_3\cdot[CH_2]_9\cdot C(=NOH)\cdot[CH_2]_6\cdot CO_2Me + CH_3\cdot[CH_2]_9\cdot C(=HON)\cdot[CH_2]_6\cdot CO_2Me$$

$$\downarrow$$

$$CH_3\cdot[CH_2]_9\cdot NH\cdot CO\cdot[CH_2]_6\cdot CO_2Me + CH_3\cdot[CH_2]_9\cdot CO\cdot NH\cdot[CH_2]_6\cdot CO_2Me$$

$$\downarrow$$

$$\underset{\text{decylamine}}{CH_3\cdot[CH_2]_9\cdot NH_2} + \underset{\text{suberic acid}}{HO_2C\cdot[CH_2]_6\cdot CO_2H} + \underset{\text{undecanoic acid}}{CH_3\cdot[CH_2]_9\cdot CO_2H} + \underset{\text{7-aminoheptanoic acid}}{NH_2\cdot[CH_2]_6\cdot CO_2H}$$

* Oxalic acid frequently results from oxidation of malonic acid.

III. The Preparation of Fatty Acids From Closely Related Compounds

Long-chain fatty acids can sometimes be obtained from closely related compounds which are more readily available, by reactions leading to the introduction or removal of unsaturated centres or by the application of standard procedures of chain extension or chain degradation. This question of availability is important; thus the preparation of stearic acid by hydrogenation of synthetic linoleic acid is of no practical importance. In so far as the closely related compounds are themselves naturally occurring fatty acids, they are acids either easily isolatable from the natural mixtures in which they occur, or present in such high proportions in some fat that the mixed acids of the latter may be used in the required reaction; castor oil with its high content of ricinoleic acid (92–95 per cent) is an example.

Examples quoted in this section will be mainly those in which the starting material either occurs naturally or is readily derived from a naturally occurring compound, but further examples of similar reactions will be found in the next section when an initial condensation product has to be converted into a desired end-product. As already stated, emphasis is laid on methods which are of preparative value.

A. Methods involving no change of chain length

1. *Hydrogenation of unsaturated acids*

By hydrogenation, unsaturated acids may be converted to their saturated homologues, polyethenoid acids to less unsaturated compounds, and acetylenic to ethylenic compounds.

Although many higher saturated acids occur naturally and may be isolated from suitable sources or obtained in other ways, it is sometimes convenient to prepare pure saturated acids from unsaturated analogues which may be more readily freed from lower or higher homologues. This method has been used to prepare undecanoic from undec-10-enoic acid (see p. 15), stearic acid from an unsaturated C_{18} acid such as elaidic or elaeostearic acid,[12] and behenic from erucic acid or its trans isomer, brassidic acid[13] (erucic acid is present in high proportions in seed fats of the Cruciferae). Catalytic reduction is preferred though chemical methods have been used.

Unsaturated acids may be prepared by partial reduction of more highly unsaturated compounds which are readily accessible. Partial reduction of polyethenoid acids, however, generally yields a complex mixture of isomers, for, in addition to the fact that different ethylenic bonds may become saturated, cis-trans isomerism and double-bond migration occur simultaneously; it is not surprising, therefore, that only a few individual compounds have been isolated.

Partial hydrogenation of sorbic acid gives hex-3-enoic acid as the main product, accompanied by appreciable quantities of hex-4-enoic acid.[14] Linoleic acid is reported to give both octadec-9- and -12-enoic acids, whilst linolenic

acid yields first diethenoid and then monoethenoid acids: one diethenoid acid (isolinoleic) has been isolated.[15, 16, 17] Elaeostearic acid is readily hydrogenated yielding trans octadec-11-enoic acid as the main but not the sole product.[8, 18, 19] Arachidonic acid is reported to give eicosa-5:14- and -8:14-dienoic acids (80–90 per cent, 5–10 per cent respectively) when partially hydrogenated.[20] The hydrogenation of methyl parinarate (methyl octadeca-9:11:13:15-tetraenoate) had also been studied.[21]

In contrast to these reactions, which are of little value for preparative purposes, though of considerable interest from other viewpoints, the partial hydrogenation of acetylenic acids is of great synthetic importance.[22, 23] The value of this reaction is enhanced by the fact that the resulting ethylenic acids are essentially cis or trans depending on the reaction conditions. Since this reaction is of great importance in those methods which make use of acetylenic intermediates, it is more conveniently discussed along with these (p. 33).

The conversion of octadec-trans-11-en-9-ynoic acid (ximenynic acid) to octadeca-trans-9-trans-11-dienoic acid (MANGOLD'S acid) illustrates the application of this partial hydrogenation to a naturally occurring compound.[24]

$$\overset{\text{trans}}{\text{—CH:CH·C}\vdots\text{C—}} \xrightarrow[\text{catalyst}]{\text{H}_2\text{-Lindlar}} \text{—CH:CH·CH:CH—} \xrightarrow{\text{I}_2\text{-sunlight}} \overset{\text{trans}}{\text{—CH:CH·}}\overset{\text{trans}}{\text{CH:CH—}}$$

2. *Oxidation of alcohols or aldehydes*

Because the alcohols or aldehydes are seldom more readily available than the corresponding acids this method has been limited to the preparation of heptanoic acid (from heptanal obtained by pyrolysis of ricinoleic acid) and the higher members of the series (above C_{25}). There has been much confusion among the higher naturally occurring alcohols. The alcohols, and consequently the resulting acids also, have often been homologous mixtures, whilst, sometimes, wrong formulae have been assigned to both alcohol and acid. PIPER, CHIBNALL, and WILLIAMS[25] in a summarizing paper describe the C_{26}, C_{28}, and C_{30} acids obtained by oxidation of the corresponding alcohols.

A method of oxidizing unsaturated alcohols to the corresponding acid in fair yield (20–40 per cent) by means of chromium trioxide—sulphuric acid—water—acetone has been reported,[26] the oxidation of non-2-en-4-ynol is typical.

$$\text{CH}_3\cdot[\text{CH}_2]_3\cdot\text{C}\vdots\text{C}\cdot\text{CH:CH}\cdot\text{CH}_2\text{OH} \rightarrow \text{CH}_3\cdot[\text{CH}_2]_3\cdot\text{C}\vdots\text{C}\cdot\text{CH:CH}\cdot\text{CO}_2\text{H}$$

3. *Preparation of unsaturated acids by methods resulting in an increase in the amount of unsaturation*

(*a*) *Dehydration of hydroxy compounds.* For the formation of individual acids this procedure suffers from the fact that dehydration frequently affords two isomers and from the limited availability of hydroxy acids; nevertheless the method has found commercial application in the production of a drying oil from castor oil.

12-Hydroxy-oleic and -stearic acids are the most readily available hydroxy acids. Dehydration of 12-hydroxystearic acid with a variety of dehydrating

agents yields a mixture of 11:12- and 12:13-unsaturated acids.[8, 27, 28] Dehydration of ricinoleic acid (I) or its glyceride affords octadecadienoic acids (II and III) or glycerides. Dehydration is effected by heating with various catalysts, and the more unsaturated product has enhanced drying properties.[29]

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H \quad \text{(II)}$$

$$CH_3\cdot[CH_2]_5\cdot CH(OH)\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H \quad \text{(I)}$$

$$CH_3\cdot[CH_2]_5\cdot CH{:}CH\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H \quad \text{(III)}$$

The claim [30] that the conjugated acid (III) is the main product has not been upheld.[31] In a recent study of the octadecadienoic acids JACKSON *et al.*[32] divided dehydrated castor oil acids into two fractions containing conjugated and nonconjugated unsaturated acids respectively, and isolated the trans-9:trans-11 and cis-9:trans-11 octadecadienoic acids from the former and the cis-9:trans-12 isomer from the latter, whilst a fourth compound isolated by SMIT[33] has been shown to be octadeca-trans-8:trans-10-dienoic acid.[34] The dehydration reaction is obviously more complex than was at first realized. The trans-9:trans-11 acid (MANGOLD's acid) also results by dehydration of ricinelaidic acid,[35] and was first prepared in this way.

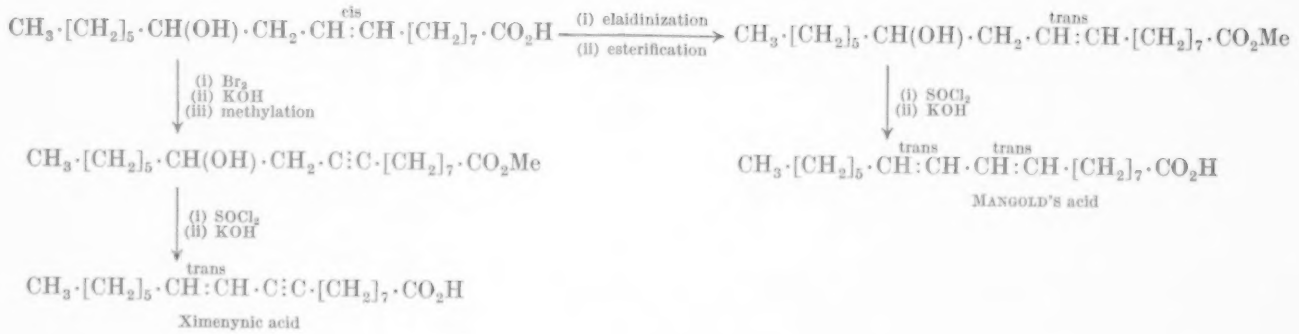
Unsaturated acids with the double bond in the end position have also been prepared from the ω-hydroxy ester by pyrolysis of the palmitoyl derivative.[36]

(*b*) *Dehydrohalogenation of halogeno compounds.* The commonest method of introducing an unsaturated linkage involves dehydrohalogenation of a halogeno compound. This reaction is generally effected by treatment with alkali or a nitrogenous base (diethylaniline, quinoline, or collidine). Alkali, although used extensively, has the disadvantage that the product may be accompanied by some hydroxy acid and by isomeric unsaturated acids resulting from double-bond migration. The required halogenated acids may be produced in four ways: (a) bromination by phosphorus and bromine giving the α-bromo acid; (b) conversion of some functional group already present (e.g. hydroxyl) into a halogen substituent; (c) interaction of a double bond with HX or X_2 (X = halogen), (d) allylic bromination of unsaturated acids by N-bromosuccinimide.

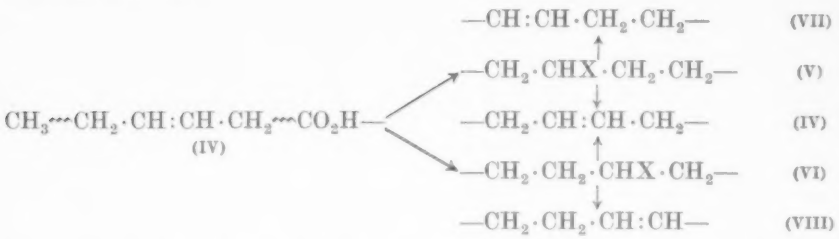
Method (a) has been widely used for the preparation of $\alpha\beta$-unsaturated acids. CASON and his colleagues[37] report potassium tert butoxide to be a satisfactory dehydrohalogenating reagent but HUNTER and POPJÁK[38] claim optimum yields for dehydrobromination of the neopentyl α-bromo ester with diethyl aniline. This elimination frequently produces a trans ethylenic system though exceptions have been reported.[39] $\alpha\beta$-unsaturated acids are also prepared by the Doebner reaction (see p. 17).

$$—CH_2\cdot CH_2\cdot CO_2R \rightarrow —CH_2\cdot CHX\cdot CO_2R \rightarrow —CH{:}CH\cdot CO_2R$$

Method (b), largely limited to the use of ricinoleic acid and 12-hydroxystearic acid, has recently been applied in a synthesis of octadec-trans-11-en-9-ynoic acid (ximenynic, santalbic acid) from ricinstearolic acid and of MANGOLD's acid from ricinoleic acid:[40]


CH3·[CH2]5·CH(OH)·CH2·CH:CH·[CH2]7·CO2H
cis
(i) elaidinization
(ii) esterification
CH3·[CH2]5·CH(OH)·CH2·CH:CH·[CH2]7·CO2Me
trans
(i) Br2
(ii) KOH
(iii) methylation
(i) SOCl2
(ii) KOH
CH3·[CH2]5·CH(OH)·CH2·C:C·[CH2]7·CO2Me
(i) SOCl2
(ii) KOH
CH3·[CH2]5·CH:CH·CH:CH·[CH2]7·CO2H
trans
trans
MANGOLD'S acid
CH3·[CH2]5·CH:CH·C:C·[CH2]7·CO2H
trans
Ximenynic acid

Unsaturated acids yield isomeric acids by hydrohalogenation and subsequent dehydrohalogenation.



On a purely random basis such a reaction would lead to three products (IV, VII, and VIII), though, in practice, one isomer may predominate and be isolatable. Factors affecting the product of this reaction include the position of the carboxyl group and other ethylenic linkages, the degree of substitution of the carbon atoms involved, and the experimental conditions. It has been claimed that (V) and (VII) are the main products and several octadecenoic acids have been prepared in this way.[41, 42, 43]

The reaction of *N*-bromosuccinimide with several unsaturated acids or esters has been examined. SCHMID and LEHMANN[44] brominated methyl elaidate and methyl brassidate in the allylic position and, after dehydrobromination, isolated octadeca-9:11-dienoic and docosa-13:15-dienoic acids. As the properties of the former differ from those of the known octadeca-trans-9:trans-11-dienoic acid, confirmation of these results is desirable. Methyl undeca-8:10-dienoate has been similarly prepared from methyl undec-10-enoate,[45] and octadeca-6:8-dienoic acid from petroselinic acid.[46] Allylic bromination of methyl linoleate and other unsaturated esters has also been studied[47, 48, 49]

Dehydrohalogenation of a saturated dihalogen-acid has been widely used for the preparation of acetylenic acids from the corresponding ethylene compounds via the dihalogen acid.[50] The dehydrohalogenating agent is generally potassium hydroxide in aqueous or alcoholic solution, though sodamide in liquid ammonia has been used. The preparation of ricinstearolic acid given above is illustrative. JOHNSON[51] states that this method cannot be used to prepare 2-ynoic acids but these are readily available by other procedures.

$$—CH:CH— \rightarrow —CHX·CHX— \rightarrow —C{:}C—$$

(c) *Dehalogenation of vicinal dihalides.* Dehalogenation of vicinal dihalides affords monounsaturated acids whilst polyunsaturated acids result from the appropriate polybromides. Being highly stereospecific this trans elimination reaction is of great value as the basis of a convenient method of preparing polyethenoid acids such as linoleic or linolenic acids. Acid mixtures rich in these compounds may be brominated and the corresponding tetra- and hexabromostearic acids readily isolated and purified. Debromination of the pure polybromides then yields the unsaturated acid having the same configuration

as originally. Traces of configurational isomers known to be present may be removed by several low-temperature crystallizations.[32, 52] Debromination is usually effected by metallic zinc or, less frequently, by iodide ions.

$$\text{—CH}^{\text{cis}}\text{:CH—} \xrightarrow[\text{trans-addition}]{\text{Br}_2} \text{—CHBr}^{\text{threo}}\text{·CHBr} \xrightarrow[\text{trans-elimination}]{\text{Zn}} \text{—CH}^{\text{cis}}\text{:CH—}$$

This reaction, coupled with the stereospecific conversion of α-glycols to dibromides (see p. 26) with inversion at one centre, has been used by BOWMAN in syntheses which proceed through an intermediate acyloin; its use is illustrated here in the partial synthesis of octadec-trans-12-enoic acid from naturally occurring 12:13-cis-epoxy oleic acid.[53]

$$\text{CH}_3\cdot[\text{CH}_2]_4\cdot\underset{\text{O}}{\text{CH}\cdot\text{CH}}\cdot\text{CH}_2\cdot\text{CH:CH}\cdot[\text{CH}_2]_7\cdot\text{CO}_2\text{H} \xrightarrow[\text{(ii) KOH}]{\text{(i) H}_2\text{—Pd—AcOH}}$$

$$\text{CH}_3\cdot[\text{CH}_2]_4\cdot\text{CH(OH)}^{\text{threo}}\cdot\text{CH(OH)}\cdot[\text{CH}_2]_{10}\cdot\text{CO}_2\text{H} \xrightarrow[-\text{H}_2\text{SO}_4]{\text{HBr—AcOH}}$$

$$\text{CH}_3\cdot[\text{CH}_2]_4\cdot\text{CHBr}^{\text{erythro}}\cdot\text{CHBr}\cdot[\text{CH}_2]_{10}\cdot\text{CO}_2\text{H} \xrightarrow{\text{Zn}}$$

$$\text{CH}_3\cdot[\text{CH}_2]_4\cdot\text{CH}^{\text{trans}}\text{:CH}\cdot[\text{CH}_2]_{10}\cdot\text{CO}_2\text{H}$$

4. *Preparation of unsaturated acids by methods occurring without change in the amount of unsaturation*

(*a*) *Change of configuration* (*elaidinization*). Ethylenic unsaturated acids can exist in cis and trans forms and since many naturally occurring acids have the cis structure it is possible to convert these into their more stable trans isomers. This change has been most fully studied in the case of oleic acid, which is converted into elaidic acid;[54] the latter name has given rise to the term elaidinization for the cis → trans change. The process is an equilibrium reaction and both oleic and elaidic acid yield the same isomeric mixture (containing *c.* 67 per cent of the trans form). Mixtures of similar composition result from other monoethenoid acids. The reagents most frequently used include the nitrogen oxides, sulphur, selenium, and iodine and/or ultra-violet light.

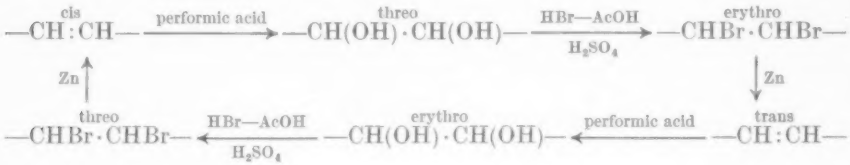
Using these reagents many unsaturated acids have been converted into their trans isomers which on account of their higher melting point and lower solubility may be separated without too much difficulty. Nonconjugated polyethenoid acids give the all trans isomer though there is evidence that linolelaidic acid[32] (trans-9:trans-12) is accompanied by the trans-9:cis-12 isomer.[55, 57]

Acids containing conjugated double bonds isomerize more readily under the influence of ultra-violet light and/or traces of iodine, and the naturally occurring acids with conjugated unsaturation containing one or more cis bonds (elaeostearic, licanic, kamlolenic, parinaric) are readily converted to their higher melting β-forms which are the all trans isomers.[58–62]

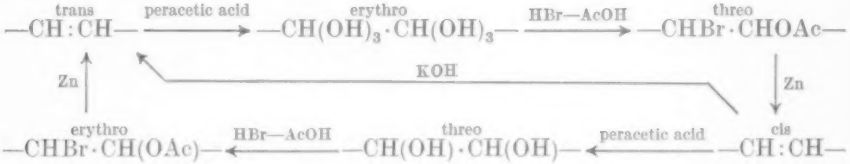
The interconversion of configurational isomers by a series of chemical reactions has been reported by MYERS,[63] by AMES and BOWMAN,[64] and by HOFF,

GREENLEE, and BOORD.[65] These are shown by the reaction sequences below; the method of HOFF *et al.* has been used for the interconversion of olefins but has not yet been applied to long-chain acids.

AMES and BOWMAN: Hendec-9-enoic acid.



MYERS: Octadec-2-enoic acid



HOFF, GREENLEE, and BOORD.

(*b*) *Migration of unsaturated centres.* The double bond(s) present in an unsaturated fatty acid may migrate under certain conditions. Thus it is possible to convert the system (IX), present in many naturally occurring acids, into the conjugated system (X) which is readily identified by its characteristic ultraviolet absorption.

(IX) —CH:CH·CH_2·CH:CH— → —CH:CH·CH:CH·CH_2— (X)

This is the basis of a quantitative method of estimating acids containing the system (IX).[66, 67] This migration, which may be accompanied by elaidinization, is usually effected by alkali at high temperatures (up to 200°) though it also occurs under conditions of hydrogenation and autoxidation.

Hex-2- and hex-3-enoic acid form an equilibrium mixture, produced from either isomer by sodium ethoxide, and containing *c.* 90 per cent of the 2-enoic acid.[68] Undec-10-ynoic acid isomerizes to undec-9-ynoic acid under strongly alkaline conditions, so that the latter is often isolated in attempts to prepare the 10 isomer from 10:11-dibromo-undecanoic acid. Fusion of oleic acid with potassium hydroxide yields palmitic acid, migration of the double bond to the $\alpha\beta$ position preceding fission.[69]

MOORE[70] has reported that prolonged hydrolysis of unsaturated fats leads to isomerization of linoleic and linolenic acids to conjugated forms. From linseed oil (containing over 60 per cent of linolenic acid) he isolated a solid acid which may be octadeca-trans-10:cis-12:trans-14-trienoic acid,[71, 72, 73] but is more probably the all trans isomer. Isomerization of dehydrated castor oil acids yields a solid mixture from which an individual acid, considered to be octadeca-trans-10:trans-12-dienoic acid,[74] can be obtained. In an interesting paper, REIMENSCHNEIDER and his co-workers[72] (see also reference 32) isolated two acids from alkali-isomerized linoleic acid which they believe to be the trans-10:cis-12- and the cis-9:trans-11-isomer. When treated with iodine these acids elaidinize to the known wholly trans isomers. The authors assume that the double bond which does not shift retains its configuration, and suggest that, when the migrating bond was trans, the new bond is either cis or trans, whilst if it was cis, the new bond is predominantly trans. The production of these octadecadienoic acids from linoleic acid by double bond migration and/or elaidinization and from ricinoleic acid by dehydration followed in some cases by double bond migration and/or elaidinization is summarized in Fig. 1. Arachidonic acid isomerizes to a mixture from which a solid acid with four conjugated double bonds has been isolated.[75]

Recent investigations have shown that acetylenic acids can be converted into allenes or, in certain favourable cases, into dienes. Since this is another method of synthesis involving acetylenic intermediates, discussion is postponed to p. 33.

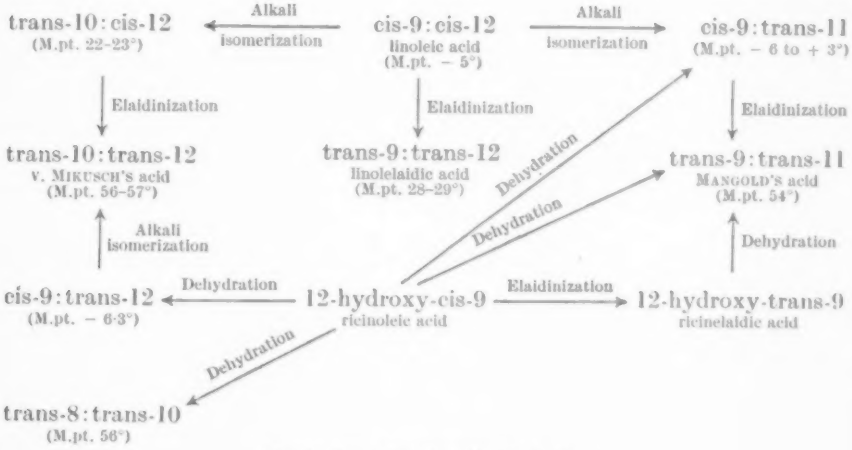


Fig. 1. Some Octadecadienoic Acids.

B. Methods involving chain degradation

There are many ways of degrading saturated or unsaturated acids to lower homologues. Although most of these have been designed for determination of structure, where yields are high and starting materials readily available, they

have also been used as preparative methods. Some of these affording homologues with one less carbon atom suffer from the disadvantage that the properties of the homologues are so similar that complete separation of the two is difficult.

1. *Reactions resulting in the loss of one carbon atom*

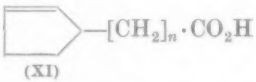
Stearic acid has been degraded stepwise to nonanoic acid by KRAFFT[76] who oxidized the methyl ketones resulting from interaction of barium acetate with the barium salt of a higher acid and this degradation was continued by HOFMANN[77, 78] who converted nonanamide to valeramide by the modification of his degradation reaction in which an amide is converted to a nitrile with one less carbon atom. Another method involves decomposition of the α-hydroxy acid by heat and subsequent oxidation or by direct oxidation;[79, 80] high yields (80–85 per cent) are claimed for the latter.

$$R\cdot CH_2\cdot CO_2Ba_{\frac{1}{2}} \xrightarrow{Ba(OAc)_2} R\cdot CH_2\cdot CO\cdot CH_3 \rightarrow R\cdot CO_2H$$

$$R\cdot CH(OH)\cdot CO_2H \xrightarrow{\text{heat}} R\cdot CHO \xrightarrow{Ag_2O} R\cdot CO_2H$$

$$R\cdot CH(OH)\cdot CO_2H \xrightarrow{KMnO_4 \text{ or } Pb(OAc)_4} R\cdot CO_2H$$

Unsaturated acids also result from degradation reactions such as the Wieland-Barbier reaction, the Hunsdiecker reaction (see p. 16), or as by-products in an electrolysis reaction. Several acids of the type $CH_2{:}CH\cdot[CH_2]_{n-2}\cdot CO_2R$ have resulted as products of a side reaction in the electrolysis of $KO_2C\cdot[CH_2]_n\cdot CO_2R$, the main product being $RO_2C\cdot[CH_2]_{2n}\cdot CO_2R$.[81–84] By Wieland-Barbier degradation oleic acids gives heptadec-8-enoic acid and hexadec-7-enoic acid,[85, 86] hendec-10-ynoic gives dec-9-ynoic acid, and hendec-10-enoic acid gives dec-10-enoic acid;[87, 88] whilst chaulmoogric acid (XI, $n = 12$) affords, after step-wise degradation, alepric acid (XI, $n = 8$),[89]



(XI)

Erucic acid, however, is stated to yield a glycol of the expected C_{21} acid.[90] The Wieland-Barbier degradation involves the following sequence of reactions:

$$R\cdot CH_2\cdot CO_2R' \xrightarrow{PhMgBr} R\cdot CH_2\cdot C(OH)Ph_2 \xrightarrow{\text{dehydration}} R\cdot CH{:}CPh_2 \xrightarrow{\text{oxidation}} R\cdot CO_2H$$

2. *Reactions resulting in the loss of more than one carbon atom*

Lower acids are readily obtained from unsaturated acids by alkali fusion or oxidation.

Whether naturally occurring or prepared from saturated acids by bromination and subsequent dehydrobromination, unsaturated acids yield the saturated acid

with two fewer carbon atoms when fused with potassium hydroxide. When the double bond is not in the $\alpha\beta$ position, migration to this position probably precedes fission. Oleic and erucic acids have long been known to give palmitic[69] and eicosanoic acid[91, 92] respectively by this means (see also reference 92a, b). HUNTER and POPJÁK[38] have used the fusion of $\alpha\beta$-unsaturated acids for the study of radioactive centres in active acids. Their complete reaction sequence, described in detail for octanoic acid and hexanoic acid, is:

$$R\cdot CH_2\cdot CH_2\cdot CO_2H \xrightarrow[Br_2]{SOCl_2} R\cdot CH_2\cdot CHBr\cdot COCl \xrightarrow[PhNEt_2]{R'OH}$$

$$R\cdot CH{:}CH\cdot CO_2R' \xrightarrow[KOH\ fusion]{hydrolysis} R\cdot CO_2H + CH_3\cdot CO_2H.$$

Optimum yields (50 per cent overall) are claimed when the neopentyl ester is dehydrobrominated with diethylaniline and by hydrolysing the ester before potash fusion.

Of the many oxidation procedures designed for fission of unsaturated acids the most satisfactory method, particularly from the preparative viewpoint, is oxidation by potassium permanganate in acetone or acetic acid solution.[7, 8]

The preparation of undec-10-enoic acid by pyrolysis of castor oil is now carried out on an industrial scale.[93–95]

C. Methods involving chain extension by one or two carbon atoms

Several of the methods now to be discussed find further applications in the condensation reactions considered later where more examples will be found.

1. *Chain extension by one carbon atom*

(*a*) Of the standard procedures used for increasing the length of a carbon chain by one unit, that involving the reaction sequence given below has been used most extensively:

$$R\cdot CO_2H \rightarrow R\cdot CO_2R' \rightarrow R\cdot CH_2OH \rightarrow R\cdot CH_2X \rightarrow R\cdot CH_2\cdot CN \rightarrow R\cdot CH_2\cdot CO_2H$$

The starting material is generally a naturally occurring acid or (less frequently) alcohol and, since these contain an even number of carbon atoms, the acids so obtained are mainly those containing an odd number of carbon atoms. Alternatively, an acid conveniently prepared by some other method may be used. The starting material must be pure if pure products are to result: indeed the main difficulty in these reactions is the separation of the products from impurities with very similar physical properties. For this reason pure stearic and behenic acid, obtained by the reduction of oleic and erucic acid, are useful starting materials. Many steps are involved but yields are good and pure products are claimed, given the above safeguard. Almost every acid in the series between C_{17} and C_{35} has been prepared in this way,[15, 96, 97] as well as some

others outside this range. A modification recently described[98] involves reaction of potassium cyanide with a tosyl (*p*-toluenesulphonyl) derivative.

$$R\cdot OTs \xrightarrow{KCN} R\cdot CN \xrightarrow{\text{hydrolysis}} R\cdot CO_2H \qquad R = HC{:}C\cdot[CH_2]_3-$$

This reaction sequence has also been used to convert hendec-10-enoic acid to dodec-11-enoic acid.[99–101]

(*b*) The Arndt-Eistert procedure for increasing chain length by one carbon atom has been used for the preparation of saturated and unsaturated acids. This reaction,[102, 103] based on WOLFF rearrangement of the diazoketone resulting from interaction of the acid chloride with diazomethane, requires a smaller number of steps than the former methods and good yields are reported. Applied to the following acids the homologues indicated in parenthesis result: hendec-10-enoic acid (C_{12}),[101] myristic acid (C_{15}),[104] palmitic acid (C_{17}),[104, 105] stearic acid (C_{19}, C_{20}, and C_{21}),[104, 106] and linoleic acid (C_{19}, C_{20}).[107]

$$R\cdot CO_2H \longrightarrow R\cdot COCl \xrightarrow{CH_2N_2} R\cdot CO\cdot CHN_2 \xrightarrow[Ag_2O]{H_2O} R\cdot CH_2\cdot CO_2H$$

(*c*) Carbonation of Grignard and other organometallic compounds yields acids with an additional carbon atom and is particularly valuable, using $C^{14}O_2$, for the preparation of acids isotopically labelled in the carboxyl group. Both saturated[108, 109] and unsaturated acids[110, 111] have been so prepared. The required halogeno compounds are conveniently obtained by the Hunsdiecker reaction involving the action of bromine on the silver salt of the acid. This is illustrated in the preparation of labelled linoleic acid:

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H \xrightarrow[\text{(i) } NH_3\text{—MeOH}]{Br_2}$$

$$CH_3\cdot[CH_2]_4\cdot CHBr\cdot CHBr\cdot CH_2\cdot CHBr\cdot CHBr\cdot[CH_2]_7\cdot CO_2H \xrightarrow[\text{(iii) } Br_2]{\text{(ii) } AgNO_2}$$

$$CH_3\cdot[CH_2]_4\cdot CHBr\cdot CHBr\cdot CH_2\cdot CHBr\cdot CHBr\cdot[CH_2]_7Br \xrightarrow{Zn}$$

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_7Br \xrightarrow[\text{(ii) } C^{14}O_2]{\text{(i) Mg}}$$

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_7\cdot C^{14}O_2H$$

BERGSTRÖM *et al.*[112] have used a modification of this reaction sequence to prepare norlinoleic acid (heptadeca-8:11-dienoic acid). The pentabromide is converted to a tetrabromoalcohol and then by oxidation and debromination to the required acid; yields of about 90 per cent are reported for each step.

2. *Chain extension by two carbon atoms*

(*a*) By using malonic ester it is possible to add two carbon atoms to the appropriate alkyl halide and this method has been used mainly to prepare the higher acids containing an even number of carbon atoms. Stearic and behenic acids are again the most important starting materials. Most of the even and some of the odd acids up to C_{38} have been prepared in this way chiefly by BLEYBERG

and ULRICH[113] and by FRANCIS *et al.*[13, 97, 114] EGLINGTON and WHITING[98] have used the tosyl derivative in place of the bromide.

$$RBr \xrightarrow[NaOEt]{CH_2(CO_2Et)_2} R\cdot CH(CO_2Et)_2 \xrightarrow{\text{hydrolysis}} R\cdot CH(CO_2H)_2 \xrightarrow{-CO_2} R\cdot CH_2\cdot CO_2H$$

Unsaturated acids, including several shorter-chain compounds, pentadec-14-enoic acid,[100] tetracos-15-enoic acid,[115] and vaccenic,[116] have also been prepared by this reaction. The product is a mixture of cis and trans isomers which must be separated by fractional crystallization.[117, 118] The production of vaccenic acid proceeds thus:

$$CH_3\cdot[CH_2]_5\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H \xrightarrow[\text{(iii) } CH_2(CO_2Et)_2 \text{ etc.}]{\text{(i) reduction of ester; (ii) } PBr_3} CH_3\cdot[CH_2]_5\cdot CH{:}CH\cdot[CH_2]_9\cdot CO_2H$$

Similar results follow when malonic ester is replaced by cyanoacetic[119] or acetoacetic ester[120] though less use has been made of these reagents in this connection.

(*b*) The Doebner reaction,[121] by which condensation of an aldehyde with malonic acid leads to a trans $\alpha\beta$-unsaturated acid,[122] has been widely used as a means of chain extension, and is frequently employed along with the condensation reactions to be described later. When the usual condensing reagent, pyridine, is replaced by dimethylaniline or triethanolamine the $\beta\gamma$-acid is said to result.[123, 124] BACHMANN[125] and TULUS[126] have described the preparation of several $\alpha\beta$-unsaturated acids and two more recent examples are shown in the following reaction sequences:

(i) Octadec-trans-2-enoic acid required as an intermediate in the preparation of dihydrosphingosine.[127]

$$CH_3\cdot[CH_2]_{14}\cdot CHO + CH_2(CO_2H_2) \xrightarrow[\text{piperidine}]{\text{pyridine}} CH_3\cdot[CH_2]_{14}\cdot \overset{\text{trans}}{CH{:}CH}\cdot CO_2H$$

(ii) Deca-trans-2-trans-4-dienoic acid used in the preparation of its isobutylamide for comparison with the insecticides pellitorine and spilanthol.[128]

$$CH_3\cdot[CH_2]_4\cdot CHO \xrightarrow[\text{pyridine}]{CH_2(CO_2H)_2} CH_3\cdot[CH_2]_4\cdot \overset{\text{trans}}{CH{:}CH}\cdot CO_2H \xrightarrow[\text{(ii) } K_2Cr_2O_7-H_2SO_4]{\text{(i) } LiAlH_4}$$

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot CHO \xrightarrow[\text{pyridine}]{CH_2(CO_2H)_2} CH_3\cdot[CH_2]_4\cdot \overset{\text{trans}}{CH{:}CH}\cdot \overset{\text{trans}}{CH{:}CH}\cdot CO_2H$$

(iii) Use has also been made of the Reformatsky reaction.[129] The condensation of an aldehyde with an α-bromo ester in presence of zinc affords a β-hydroxy ester readily dehydrated to the $\alpha\beta$-unsaturated analogue. Chain length is increased by two carbon atoms using ethyl α-bromoacetate, whilst homologues of this latter usually afford branched-chain esters. Methyl bromocrotonate, however, as the vinylogue of the α-bromoacetate gives a straight-chain compound containing four carbon atoms more than the original aldehyde. These reactions are illustrated by the following two examples.[127, 130]

$$CH_3\cdot[CH_2]_{14}\cdot CHO \xrightarrow{BrCH_2\cdot CO_2Et} CH_3\cdot[CH_2]_{14}\cdot CH(OH)\cdot CH_2\cdot CO_2H$$

$$\xrightarrow{pCH_3\cdot C_6H_4\cdot SO_2H} CH_3\cdot[CH_2]_{14}\cdot \overset{\text{trans}}{CH}:CH\cdot CO_2H$$

$$CH_3\cdot[CH_2]_n\cdot CHO \xrightarrow{BrCH_2\cdot CH:CH\cdot CO_2Me} CH_3\cdot[CH_2]_n\cdot CH(OH)\cdot CH_2\cdot CH:CH\cdot CO_2Me$$

(n = 7, 8, 9, 10, 12)

IV. The Preparation of Fatty Acids by Union of Smaller Molecules

Having discussed the preparation of fatty acids by the modification of closely related compounds attention is now directed to those methods by which long-chain compounds are built up from smaller molecules. Reactions have been developed which lead to the production of either saturated or unsaturated acids and it is in the syntheses of the latter that most advance has been made in the last decade.

It will be observed that conversion of the initial condensation product into the desired acid is frequently a similar problem to that discussed in the previous section, viz. that of modifying a closely related compound and further examples of the methods just elaborated will be found in the ensuing pages.

A. Condensations involving the use of ethyl acetoacetate or malonic ester as a coupling unit

1. *Methods using ethyl acetoacetate*

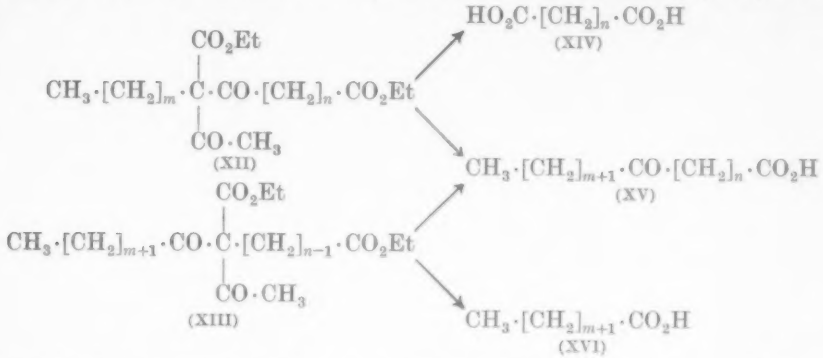
In 1925 Robinson and Robinson[131] described a method of preparing long-chain keto-acids involving condensation of (i) an alkyl halide and (ii) a carbethoxy-acyl halide with acetoacetic ester. Subsequent stepwise hydrolysis afforded a keto acid which could be reduced by the Clemmensen procedure to

$$\begin{array}{l}CO_2Et\\|\\CH_2\\|\\CO\cdot CH_3\end{array} \xrightarrow{CH_3\cdot[CH_2]_5Br} CH_3\cdot[CH_2]_6\cdot\begin{array}{l}CO_2Et\\|\\CH\\|\\CO\cdot CH_3\end{array} \xrightarrow{Cl\cdot CO\cdot[CH_2]_8\cdot CO_2Et}$$

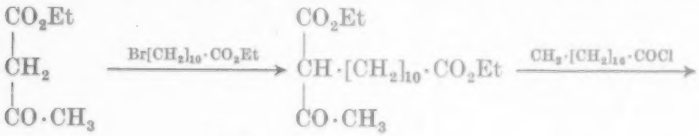
$$CH_3\cdot[CH_2]_6\cdot\begin{array}{l}CO_2Et\\|\\C\\|\\CO\cdot CH_3\end{array}\cdot CO\cdot[CH_2]_8\cdot CO_2Et \rightarrow CH_3\cdot[CH_2]_7\cdot CO\cdot[CH_2]_8\cdot CO_2H$$

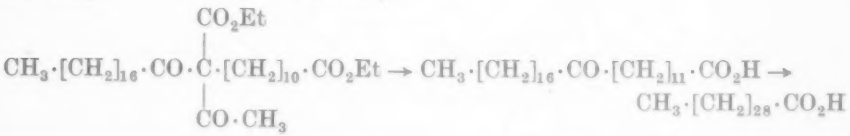
the saturated fatty acid. One author later drew attention to the rather low yields obtained.[132] The main product appears to be a dibasic acid (XIV), produced by the alternative hydrolysis of the condensation product (XII). Other work on the hydrolysis of diketones of the type $R\cdot CO\cdot CH_2\cdot CO\cdot R'$ shows that the

stronger acid preponderates. The desired keto-acid (XV) may result from either (XII) or (XIII), and since (XIV) is a stronger acid than (XVI) it was



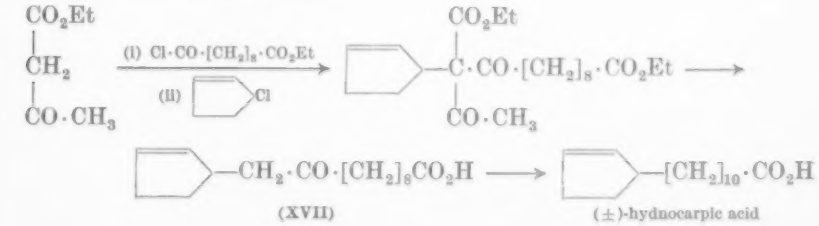
conceived that (XIII) might give a better yield of the acid (XV). This was confirmed for the preparation of several acids.[114, 133, 134] The method is





limited only by the availability of ω-bromo esters; the C_{10} and C_{11} compounds were accessible when this work was described, thus permitting the addition of eleven or twelve carbon atoms in one cycle of reactions, and other ω-bromo esters are now available. By these methods several keto acids, the largest being a C_{46} compound, have been prepared and some have been reduced to the saturated analogues.[131–134]

Although used mainly for keto (and thence saturated) acids this procedure has also been used for a few unsaturated acids including two unsaturated keto acids,[132] octadec-16 and 17-enoic acids,[135] (±) chaulmoogric acid,[136] and (±) hydnocarpic acid.[137] This last is detailed for illustration. The intermediate keto-acid (XVII) isolated as its semicarbazone, affords (±)-hydnocarpic acid on heating with sodium ethoxide.



An improved modification due to STÄLLBERG-STENHAGEN and STENHAGEN *et al.* which has given excellent results, involves condensation between an acid chloride and acetoacetic ester followed by hydrolysis to give a new β-keto ester which is then condensed with an ω-halogeno ester. Many normal and branched chain acids have been prepared in this way and the following is typical.[138]

$$CH_3\cdot[CH_2]_{20}\cdot COCl \xrightarrow{CH_3\cdot CO\cdot CH_2\cdot CO_2Et} CH_3\cdot[CH_2]_{20}\cdot CO\cdot \underset{\displaystyle CO\cdot CH_3}{\overset{|}{C}}H\cdot CO_2Et \xrightarrow{NaOMe}$$

$$CH_3\cdot[CH_2]_{20}\cdot CO\cdot CH_2\cdot CO_2Me \xrightarrow{I[CH_2]_{10}\cdot CO_2Et}$$

$$CH_3\cdot[CH_2]_{20}\cdot CO\cdot \underset{\displaystyle CO_2Me}{\overset{|}{C}}H\cdot[CH_2]_{10}\cdot CO_2Et \xrightarrow[\text{(ii) reduction}]{\text{(i) hydrolysis, } -CO_2} CH_3\cdot[CH_2]_{32}\cdot CO_2H$$

n-tetratriacontanoic acid

A recent and interesting application of this reaction has led to the preparation of the trans-9:10 and 11:12-methylene-octadecanoic acids.[139] Starting with trans-cyclo-propane-1:2-dicarboxylic acid, two side chains are built on by successive applications of these chain extending reactions. The flow sheet indicates the sequence of reactions:

$$HO_2C\cdot \underset{\displaystyle CH_2}{\overset{/\backslash}{C}}H\cdot CH\cdot CO_2H \longrightarrow ClOC\cdot \underset{\displaystyle CH_2}{\overset{/\backslash}{C}}H\cdot CH\cdot CO_2Me \xrightarrow{CH_3\cdot CO\cdot CH_2\cdot CO_2Et}$$

$$EtO_2C\cdot \underset{\displaystyle CH_3\cdot CO}{\overset{|}{C}}H\cdot CO\cdot \underset{\displaystyle CH_2}{\overset{/\backslash}{C}}H\cdot CH\cdot CO_2Me \xrightarrow{NaOMe—MeOH}$$

$$MeO_2C\cdot CH_2\cdot CO\cdot \underset{\displaystyle CH_2}{\overset{/\backslash}{C}}H\cdot CH\cdot CO_2Me \xrightarrow[\text{(ii) hydrolysis, } -CO_2]{\text{(i) } CH_3\cdot[CH_2]_nI(n = 3 \text{ or } 5)}$$

$$CH_3\cdot[CH_2]_n\cdot CH_2\cdot CO\cdot \underset{\displaystyle CH_2}{\overset{/\backslash}{C}}H\cdot CH\cdot CO_2Me \xrightarrow[\text{reduction}]{\text{Huang Minlon}}$$

$$CH_3[CH_2]_{n+2}\cdot \underset{\displaystyle CH_2}{\overset{/\backslash}{C}}H\cdot CH\cdot CO_2H \xrightarrow[\text{(ii) } CH_3\cdot CO\cdot CH_2\cdot CO_2Et\\ \text{(iii) NaOMe—MeOH}]{\text{(i) } SOCl_2}$$

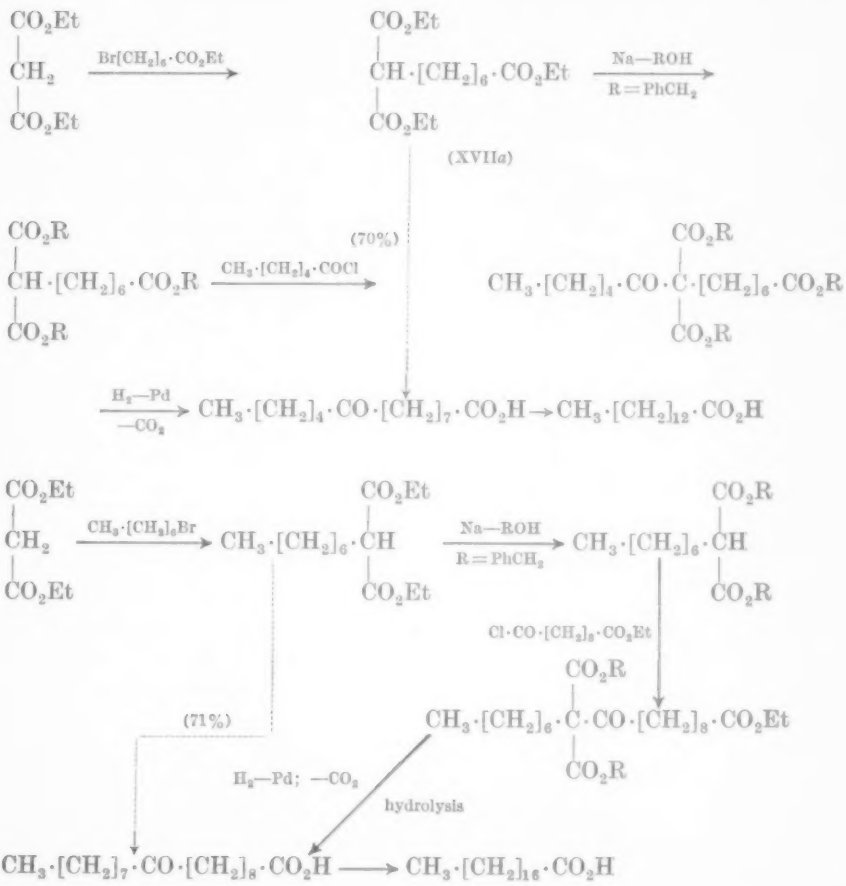
$$CH_2\cdot[CH_2]_{n+2}\cdot \underset{\displaystyle CH_2}{\overset{/\backslash}{C}}H\cdot CH\cdot CO\cdot CH_2\cdot CO_2Me \xrightarrow[\text{(ii) hydrolysis, } -CO_2\\ \text{(iii) reduction}]{\text{(i) } I[CH_2]_m\cdot CO_2Et(m = 7 \text{ or } 5)}$$

$$CH_3\cdot[CH_2]_{n+2}\cdot \underset{\displaystyle CH_2}{\overset{/\backslash}{C}}H\cdot CH\cdot[CH_2]_{m+2}\cdot CO_2H$$

By making use of hendec-10-enoic acid, STENHAGEN[140] has prepared an unsaturated acid, tricos-22-enoic acid, in this way.

2. *Methods using malonic ester*

BOWMAN and his colleagues[141, 142] have recently described methods, similar to those of ROBINSON, but using malonic ester as the coupling unit. The novelty of the method is in the use of the benzyl esters, since the benzyl group may be



split off by hydrogenation, thus avoiding the hydrolysis by which undesirable by-products are formed. The benzyl esters are prepared from the ethyl esters by transesterification. Yields are high and the desired intermediates are readily prepared. The reaction may be carried out in two ways (see reaction chart). In addition to these acids (C_{14} and C_{18}) the C_{23}, C_{39}, and C_{56} acids have been prepared. The preparation of ω-bromoheptanoic acid from tetrahydropyran

(60 per cent) is described, also an alternative preparation of triesters of type (XVIIa) via the glyoxalate thus:

$$EtO_2C\cdot CH_2\cdot[CH_2]_n\cdot CO_2Et \xrightarrow{(CO_2Et)_2} EtO_2C\cdot \underset{\displaystyle CO\cdot CO_2Et}{\overset{|}{C}}H\cdot[CH_2]_n\cdot CO_2Et \xrightarrow[-CO]{\text{heat}} (EtO_2C)_2CH\cdot[CH_2]_n\cdot CO_2Et.$$

The application of these procedures to unsaturated acids is discussed separately on pp. 27 and 28.

B. Condensations using organometallic compounds

For convenience of classification, reactions of the organometallic derivatives of acetylenic compounds are excluded from this section which is thus concerned mainly with the use of Grignard reagents or of cadmium or zinc dialkyls. These have been used in the preparation of saturated and unsaturated acids.

Fatty acids result, via the keto-esters, from interaction of a carbethoxy-acyl halide with an organometallic compound, generally a zinc or cadmium compound. These methods,[143] which have found extensive use in the preparation of branched-chain compounds,[144] are summarized thus:

$$RX \rightarrow RMgX \xrightarrow[ZnCl_2]{CdCl_2} \begin{matrix} R_2Cd \\ RZnX \end{matrix} \xrightarrow{Cl\cdot CO\cdot[CH_2]_n\cdot CO_2Et} R\cdot CO\cdot[CH_2]_n\cdot CO_2Et \rightarrow R\cdot[CH_2]_{n+1}\cdot CO_2H$$

The C_{26}–C_{35} straight-chain acids have been prepared in this way.[145–148]

The method has been extended to the preparation of dibasic acids[149] but has been little used for preparation of unsaturated acids, though HUBER[150] has obtained octadec-17-enoic acid by this means. The important reaction is between diundec-10-enylcadmium and 6-carbethoxyhexanoyl chloride.

$$Cd([CH_2]_9\cdot CH:CH_2)_2 + 2Cl\cdot CO\cdot[CH_2]_5\cdot CO_2Et \rightarrow 2CH_2:CH\cdot[CH_2]_9\cdot CO\cdot[CH_2]_5\cdot CO_2Et$$

Interaction of a Grignard compound with an aldehydo ester affords a hydroxy ester. This reaction has been used mainly to prepare unsaturated acids, but FIESER and SZMUSZKOVICZ[151] have indicated how saturated acids result from alicyclic ketones.

$$RMgX + O{:}\underset{\displaystyle [CH_2]_n}{\overset{|}{C}} \longrightarrow R{-}\underset{\displaystyle [CH_2]_n}{\overset{|}{\overset{\displaystyle HO}{\overset{|}{C}}}} \xrightarrow{CrO_3\text{—}AcOH} R\cdot CO\cdot[CH_2]_{n-1}\cdot CO_2H \xrightarrow[\text{reduction}]{\text{Huang Minlon}} R\cdot[CH_2]_n\cdot CO_2H$$

The method was illustrated by the description of the C_{20}, C_{22}, C_{24}, and C_{26} acids.

DELABY *et al.*[152, 153] used the Grignard reagent to prepare several unsaturated acids. Interaction of an alkylmagnesium halide with acraldehyde gave a vinyl secondary alcohol (XVIII) which on bromination affords the compound (XIX). This yields an unsaturated acid via the alcohol (Δ^2) or the nitrile (Δ^3). Acids of the C_6—C_9 series have been so prepared.

$$CH_3\cdot[CH_2]_n\cdot MgX + CH_2{:}CH\cdot CHO \rightarrow CH_3\cdot[CH_2]_n\cdot \underset{\text{(XVIII)}}{CH(OH)\cdot CH{:}CH_2}$$

$$\downarrow PBr_3$$

$$CH_3\cdot[CH_2]_n\cdot \underset{\text{(XIX)}}{CH{:}CH\cdot CH_2Br} \leftrightharpoons CH_3\cdot[CH_2]_n\cdot CHBr\cdot CH{:}CH_2$$

$$\downarrow NaOAc$$

$$CH_3\cdot[CH_2]_n\cdot CH{:}CH\cdot CH_2OAc \xrightarrow[\text{(ii) oxidation}]{\text{(i) hydrolysis}} CH_3\cdot[CH_2]_n\cdot CH{:}CH\cdot CO_2H$$

(CuCN)

$$CH_3\cdot[CH_2]_n\cdot CH{:}CH\cdot CH_2\cdot CN \xrightarrow{\text{hydrolysis}} CH_3\cdot[CH_2]_n\cdot CH{:}CH\cdot CH_2\cdot CO_2H$$

The most important syntheses in this section are those affording unsaturated acids by application of the BOORD synthesis of olefins.[154] Though better methods are now available, this represents the first systematic approach to the synthesis of unsaturated acids. Basically the reaction consists of condensation of a Grignard reagent with a 1-methoxy-1:2-dibromide to give a vicinal methoxy-bromo compound, subsequent removal of these groups giving a double bond:

$$R\cdot CHBr\cdot CHBr\cdot OMe + BrMgR' \rightarrow R\cdot CHBr\cdot CH(OMe)\cdot R' \rightarrow R\cdot CH{:}CH\cdot R'$$

Using suitable compounds the Δ^9, Δ^{11}, Δ^{15}, and $\Delta^{9\ 12}$ octadecenoic acids have been prepared. A great disadvantage, in comparison with more recent methods, is the lack of stereochemical control over the configuration of the double bond; cis and trans isomers have to be isolated from the mixed reaction product.

In 1934 NOLLER and BANNEROT[155] described the synthesis of oleic and elaidic acids. Condensation of 1:2-dibromo-9-chloro-1-methoxynonane (XX) with octyl magnesium bromide yielded 8-bromo-1-chloro-9-methoxyheptadecane (XXI); removal of the 8:9 substituents by treatment with zinc then gave a double bond in this position, and the chloride was converted into the acid via the nitrile. The final product was a mixture of elaidic and oleic acids. Even this is not a complete synthesis since the chlorononanal was obtained from butyl oleate.

$$Cl[CH_2]_7\cdot CH_2\cdot CHO \xrightarrow[\text{(ii) MeOH—HBr}]{\text{(i) } Br_2} \underset{\text{(XX)}}{Cl[CH_2]_7\cdot CHBr\cdot CHBr\cdot OMe}$$

$$\xrightarrow{BrMg\cdot[CH_2]_7\cdot CH_3} \underset{\text{(XXI)}}{Cl[CH_2]_7\cdot CHBr\cdot CH(OMe)\cdot[CH_2]_7\cdot CH_3} \xrightarrow[\text{(iii) hydrolysis}]{\text{(i) Zn; (ii) NaCN}}$$

$$HO_2C\cdot[CH_2]_7\cdot CH{:}CH\cdot[CH_2]_7\cdot CH_3$$

NOLLER and GIRVIN[156] later attempted a similar synthesis of linoleic acid by the annexed scheme. The product, obtained in poor yield, gave no tetrabromostearic acid on bromination, though a little tetrahydroxystearic acid was isolated after oxidation, indicating the presence of some linoleic acid.

$$CH_3\cdot[CH_2]_4\cdot MgBr + CH_2{:}CH\cdot CHO \rightarrow CH_3\cdot[CH_2]_4\cdot CH(OH)\cdot CH{:}CH_2 \xrightarrow{PBr_3}$$

$$CH_3\cdot[CH_2]_4\cdot CHBr\cdot CH{:}CH_2 \rightleftharpoons$$

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot CH_2Br \xrightarrow{\text{(i) Mg; (ii) } Cl[CH_2]_7\cdot CHBr\cdot CHBr\cdot OMe}$$

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot CH_2\cdot CH(OMe)\cdot CHBr\cdot[CH_2]_7Cl \xrightarrow[\text{(iii) hydrolysis}]{\text{(i) Zn; (ii) NaCN}}$$

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H$$

BAUDART has also prepared (i) oleic and elaidic acids[157] and (ii) linolelaidic acid[158] by a slight modification of this process. The monoethenoid acids resulted from the following reaction sequence:

$$CH_3\cdot[CH_2]_7\cdot CH_2\cdot CHO \xrightarrow[\text{(ii) } BrMg\cdot[CH_2]_6\cdot OMe]{\text{(i) } Br_2\text{—EtOH}}$$

$$CH_3\cdot[CH_2]_7\cdot CHBr\cdot CH(OEt)\cdot[CH_2]_6\cdot OMe \xrightarrow{Zn\text{—BuOH}}$$

$$CH_3\cdot[CH_2]_7\cdot CH{:}CH\cdot[CH_2]_6\cdot OMe \xrightarrow[\text{(iii) Zn}]{\text{(i) } Br_2\text{; (ii) HBr}}$$

$$CH_3\cdot[CH_2]_7\cdot CH{:}CH\cdot[CH_2]_6Br \xrightarrow[\text{(ii) } CH_2(CO_2Et)_2 \text{ etc.}]{\text{(i) NaI}}$$

$$CH_3\cdot[CH_2]_7\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H$$

The starting material (XXII) for the linolelaidic acid preparation was obtained from glutaraldehyde, and the final product yielded a little tetrabromostearic acid identical with that obtained from linolelaidic acid.

$$\underset{\text{(XXII)}}{BrCH(OEt)\cdot CHBr\cdot CH_2\cdot CHBr\cdot CHBr(OEt)} \xrightarrow[\text{(ii) } BrMg\cdot[CH_2]_6\cdot OMe]{\text{(i) } CH_3\cdot[CH_2]_4\cdot MgBr}$$

$$CH_3\cdot[CH_2]_4\cdot CH(OEt)\cdot CHBr\cdot CH_2\cdot CHBr\cdot CH(OEt)\cdot[CH_2]_6\cdot OMe \xrightarrow[\text{(iii) HBr; (iv) Zn}]{\text{(i) Zn; (ii) } Br_2}$$

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_6Br \xrightarrow[\text{(ii) } CH_2(CO_2Et)_2 \text{ etc.}]{\text{(i) NaI}}$$

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H$$

TOYAMA and YAMAMATO[159] have used this method to prepare hexadec-13-enoic acid and octadec-15-enoic acid, the required ω-chlorotridecanal being obtained from ethyl erucate.

$$CH_3\cdot[CH_2]_7\cdot CH{:}CH\cdot[CH_2]_{11}\cdot CO_2Et \xrightarrow[\text{(iii) } CH_3CO_3H\text{; (iv) } Pb(OAc)_4]{\text{(i) Na—BuOH; (ii) } SOCl_2\text{—pyridine}}$$

$$OHC\cdot[CH_2]_{12}Cl \xrightarrow[\text{(ii) HBr—MeOH}]{\text{(i) } Br_2\text{—}CCl_4} MeO\cdot CHBr\cdot CHBr\cdot[CH_2]_{11}Cl$$

$$\xrightarrow[\text{(iii) KCN; (iv) hydrolysis}]{\text{(i) EtMgBr; (ii) Zn}} CH_3\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_{11}\cdot CO_2H$$

$$\xrightarrow[\text{(iii) HBr; (iv) } CH_2(CO_2Et)_2 \text{ etc.}]{\text{(i) Esterify; (ii) Na—BuOH}} CH_3\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_{13}\cdot CO_2H$$

Elaidic and vaccenic (trans-octadec-11-enoic) acids have been prepared by another modification (shown below) in which the double bond is already present in one of the starting materials. The products were contaminated with their cis isomers and with a vinylpalmitic acid resulting from reaction of the Grignard complex with (XXIV). These authors[161] have reported an improved synthesis of vaccenic acid in which 11-chloro-undecene replaces the ester (XXIII), the reaction sequence being completed by malonation.

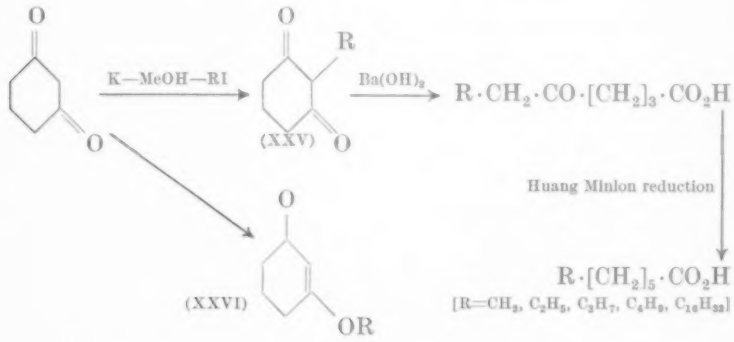
$$\underset{\substack{(n = 7 \text{ or } 9)\\(\text{XXIII})}}{CH_2{:}CH\cdot CH_2\cdot[CH_2]_n\cdot CO_2Me} \xrightarrow{C_2H_4(CO)_2NBr} \underset{(\text{XXIV})}{CH_2{:}CH\cdot CHBr\cdot[CH_2]_n\cdot CO_2Me} \rightleftharpoons$$

$$BrCH_2\cdot CH{:}CH\cdot[CH_2]_n\cdot CO_2Me \xrightarrow[m = 6 \text{ or } 4]{CH_3\cdot[CH_2]_m\cdot MgBr} CH_3\cdot[CH_2]_m\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_n\cdot CO_2Me$$

C. The use of cyclic compounds as chain extenders

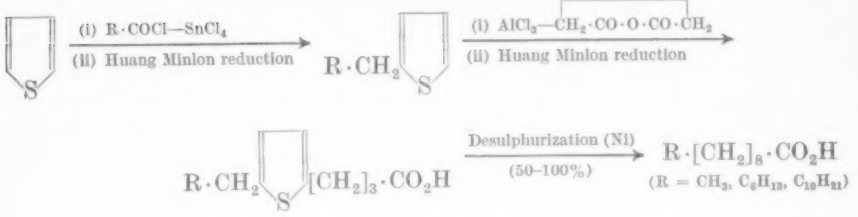
Reference has already been made to the use of cyclic ketones in the preparation of aliphatic acids (p. 22) and some further examples of the use of cyclic compounds will now be discussed.

A new method of extending chain length by six carbon atoms has been described by Stetter and Dierichs.[162] Alkylation of cyclohexane-1:3-dione affords a mixture of the C-alkyl (XXV) and O-alkyl (XXVI) derivatives; hydrolysis of the former then yields a keto acid which may be reduced to the saturated acid. The authors have also combined these two steps into a single



process[163] by the use of sodium or sodium hydroxide, hydrazine hydrate, and diethylene glycol. Many mono- and di-basic acids have been so prepared, mostly with branched chains;[164] non-8-enoic acid results from the use of allyl bromide in the above reaction.[164a]

Badger, Rodda, and Sasse[165] have made use of the special reactions of thiophene to prepare straight-chain and branched-chain fatty acids.

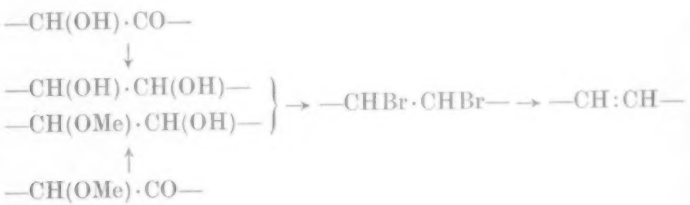


Thiophene is twice acylated and subsequently reduced (Huang Minlon) first with an acyl halide and secondly with succinic anhydride, and then desulphurized, when a saturated aliphatic acid results.

Similar reactions are described by PAPA, SCHWENK, and GINSBERG[166] and by GREY, MCGHIE, PRADHAN, and ROSS.[167] This method is probably capable of wide application.

D. Condensations yielding acyloins or alkoxyketones and thence unsaturated acids

A successful method of preparing monothenoid acids makes use of acyloins or alkoxyketones as intermediates. The former may be prepared by RUZICKA's acyloin synthesis[168] or, alternatively, both may be prepared by BOWMAN's ketone synthesis.[169] In either case the intermediate is readily converted into an unsaturated compound via the dibromide thus:



An advantage of the acyloin method is that the α-glycol may be separated into threo and erythro forms which subsequently afford the trans and the cis unsaturated acid respectively. Much of the success of this method lies in the smooth conversion of glycol to dibromide and thence to olefin, both reactions being highly stereospecific. Conversion of glycol to dibromide is effected by hydrogen bromide—acetic acid—conc. sulphuric acid and occurs with inversion; debromination, generally brought about by activated zinc, is a trans elimination. The threo glycol thus gives the trans acid via the erythro dibromide and the erythro glycol gives the cis acid via the threo dibromide.[170] In general, excellent results are obtained but in some cases the alkoxyketone procedure is preferable. AMES and BOWMAN[170] have compared the two methods.

BAUDART,[171] using RUZICKA's acyloin synthesis, prepared the cis and the trans forms of octadec-9-enoic acid and hexadec-9-enoic acid thus:

$$CH_3\cdot[CH_2]_n\cdot CO_2Et + EtO_2C.[CH_2]_7\cdot OEt \xrightarrow{Na}$$
(n = 5 or 7)

$$CH_3\cdot[CH_2]_n\cdot CO\cdot CH(OH)\cdot[CH_2]_7\cdot OEt \xrightarrow{Ni—H_2}$$

$$CH_3\cdot[CH_2]_n\cdot CH(OH)\cdot CH(OH)\cdot[CH_2]_7\cdot OEt \xrightarrow[(ii)\ Zn]{(i)\ HBr—AcOH—H_2SO_4}$$
(two isomers separated)

$$CH_3\cdot[CH_2]_n\cdot CH{:}CH\cdot[CH_2]_7Br \xrightarrow[(ii)\ hydrolysis]{(i)\ KCN}$$

$$CH_3\cdot[CH_2]_n\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H$$

In the preparation of hendec-5-enoic acid the ethoxy ester was replaced by an ω-unsaturated ester, subsequent oxidation of which gave the desired acid.

$$CH_3\cdot[CH_2]_4\cdot CO_2Et + EtO_2C\cdot[CH_2]_3\cdot CH{:}CH_2 \xrightarrow{Na}$$

$$CH_3\cdot[CH_2]_4\cdot CO\cdot CH(OH)\cdot[CH_2]_3\cdot CH{:}CH_2 \xrightarrow{Al(OPr^i)_3}$$

$$CH_3\cdot[CH_2]_4\cdot CH(OH)\cdot CH(OH)\cdot[CH_2]_3\cdot CH{:}CH_2 \xrightarrow[(iii)\ oxidation]{(i)\ Ac_2O;\ (ii)\ O_3}$$
(two isomers separated)

$$CH_3\cdot[CH_2]_4\cdot CH(OAc)\cdot CH(OAc)\cdot[CH_2]_3\cdot CO_2H \xrightarrow[(ii)\ HBr;\ (iii)\ NaI—Zn]{(i)\ hydrolysis}$$

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot[CH_2]_3\cdot CO_2H$$

Bowman and his collaborators[170, 172, 173] have prepared tetradec-9-enoic, hexadec-9-enoic, heptadec-9-enoic, octadec-4-enoic, octadec-9-enoic, eicos-9-enoic, and docos-13-enoic acids. In each case pure cis and trans isomers were obtained. The details of this procedure have already been discussed (p. 21) and two examples only are given in the annexed schemes.

Acyloin

$$CH_3\cdot[CH_2]_3\cdot CH(OAc)\cdot COCl + Na\cdot C(CO_2R)_2\cdot[CH_2]_6\cdot CO_2R \longrightarrow$$
(R = $PhCH_2$)

$$CH_3\cdot[CH_2]_3\cdot CH(OH)\cdot CO\cdot C(CO_2R)_2\cdot[CH_2]_6\cdot CO_2R \xrightarrow[(iii)\ cat.\ redn.]{(i)\ hydrogenolysis\ (ii)\ decarboxylation}$$

$$CH_3\cdot[CH_2]_3\cdot CH(OH)\cdot CH(OH)\cdot[CH_2]_7\cdot CO_2H \xrightarrow[(ii)\ Zn]{(i)\ HBr—AcOH—H_2SO_4}$$
(Two isomers separated)

$$CH_3\cdot[CH_2]_3\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H$$

Alkoxyketone

$$CH_3\cdot[CH_2]_7\cdot CH_2\cdot CO_2H \xrightarrow[\text{(iii) MeOH}]{\text{(i) } SOCl_2\text{; (ii) } Br_2} CH_3\cdot[CH_2]_7\cdot CHBr\cdot CO_2Me$$

$$\xrightarrow[\text{(ii) } Ag_2O\text{—MeI}]{\text{(i) NaOMe—MeOH}} CH_3\cdot[CH_2]_7\cdot CH(OMe)\cdot CO_2Me \xrightarrow[\text{(ii) } SOCl_2]{\text{(i) hydrolysis}}$$

$$CH_3\cdot[CH_2]_7\cdot CH(OMe)\cdot COCl + Na\cdot C(CO_2R)_2\cdot[CH_2]_{10}\cdot CO_2R \longrightarrow$$

(R = $PhCH_2$)

$$CH_3\cdot[CH_2]_7\cdot CH(OMe)\cdot CO\cdot C(CO_2R)_2\cdot[CH_2]_{10}\cdot CO_2R \xrightarrow[\text{(iii) } Al(OPr^i)_3]{\text{(i) hydrogenolysis; (ii) decarboxylation}}$$

$$CH_3\cdot[CH_2]_7\cdot CH(OMe)\cdot CH(OH)\cdot[CH_2]_{11}\cdot CO_2H \xrightarrow[\text{(ii) Zn}]{\text{(i) HBr—AcOH—}H_2SO_4}$$

$$CH_3\cdot[CH_2]_7\cdot CH{:}CH\cdot[CH_2]_{11}\cdot CO_2H$$

E. Anodic syntheses

This method of preparing long-chain acids is one of the simplest and most versatile and, chiefly in the hands of LINSTEAD and WEEDON and their co-workers, has been applied to the synthesis of saturated, ethylenic, acetylenic, and dihydroxy acids. These authors, and many others, have also applied this method to the production of branched-chain compounds.

About a century ago KOLBE[174] showed that electrolysis of an aqueous solution of an alkali metal carboxylate gave a hydrocarbon and carbon dioxide, sometimes accompanied by the products of side reactions. Dibasic acids do not

$$2C_nH_{2n+1}CO_2^- \rightarrow 2CO_2 + C_{2n}H_{4n+2}$$

react in this way but the half esters of aliphatic dibasic acids react smoothly to give diesters.[175, 176]

$$2RO_2C\cdot[CH_2]_n\cdot CO_2^- \rightarrow 2CO_2 + RO_2C\cdot[CH_2]_{2n}\cdot CO_2R$$

Electrolysis of a mixture of acids affords a mixture of products including that formed by "crossed" coupling. By using the half ester of an aliphatic dibasic acid and an aliphatic monobasic acid the product of "crossed" coupling is a longer chain ester. If appropriate compounds are chosen, then the desired ester can be readily separated from the hydrocarbon and diester resulting from symmetrical coupling. The yield is not very great (30–40 per cent) but this is more than compensated for by the very direct procedure.

$$R\cdot CO_2H + HO_2C\cdot[CH_2]_n\cdot CO_2Me \rightarrow R\cdot R + MeO_2C\cdot[CH_2]_{2n}\cdot CO_2Me + R\cdot[CH_2]_n\cdot CO_2Me$$

It has been shown that branched-chain compounds can be used in this reaction so long as the branching is not α to the carboxyl group and that ethylenic or acetylenic compounds may be used so long as the unsaturation is not $\alpha\beta$ or $\beta\gamma$ to the carboxyl group. If these conditions are satisfied the optical activity of an asymmetric centre or the configuration of an ethylenic bond will be retained in the reaction. Practical details are given in the papers of LINSTEAD and WEEDON *et al.* and the method has been reviewed by the latter author.[177]

Some examples are given in Table 1 and from these it is clearly seen that saturated acids may often be prepared from different combinations of mono- and di-basic acids and that acetylenic compounds may be used and subsequently converted to the ethylenic compounds by partial reduction (see p. 33). When the monobasic acid required as starting material is not easily purified, this may be replaced by the appropriate dihydroxy acid and the product subsequently converted to the ethylenic compound via the dibromide (see p. 26). The use of benzyl esters is sometimes advantageous.

F. The preparation and use of acetylenic intermediates

The chemistry of acetylenic compounds has developed greatly in recent years and the results have found application in many fields including the synthesis of long-chain compounds. The methods already described in this chapter have yielded, almost without exception, acids no more complex than those with one unsaturated centre, but by the use of acetylenic intermediates structures as complex as those of linoleic and erythrogenic acid have been obtained.

$$CH_3\cdot[CH_2]_4\cdot CH{:}CH\cdot CH_2\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H$$

linoleic acid

$$CH_2{:}CH\cdot[CH_2]_4\cdot C{\vdots}C\cdot C{\vdots}C\cdot[CH_2]_7\cdot CO_2H$$

erythrogenic acid

Since these developments are so recent it will be convenient, before giving examples of syntheses, to indicate briefly those reactions of acetylenic compounds which have been most used in this connection. These are of two types viz. (i) the condensation reactions of acetylenic compounds by which the desired carbon chains are produced and (ii) those reactions peculiar to acetylenic compounds by which the acetylenic intermediate is converted to the desired acid. Necessarily this account must be brief but fuller details may be found in RAPHAEL'S excellent monograph[184] which gives a critical description of the preparation and use of acetylenic compounds.

1. *Condensation reactions of acetylenic compounds*

Of chief interest here are the use of alkali metal derivatives and Grignard complexes, and an oxidative coupling reaction.

(*a*) Acetylene and acetylenic compounds containing ethynyl hydrogen react

Table 1. Straight-chain fatty acids prepared by anodic synthesis

*Monobasic acid**	*Dibasic acid† (half ester)* $HO_2C\cdot[CH_2]_n\cdot CO_2R$	*Product (after hydrolysis)*	*Yield, %*	*Ref.*
	(i) *Saturated Acids*			
C_2	4	C_6	30–40	(178)
C_4	4	C_8	30–40	(178)
C_6	4	C_{10}	35–60	(178)
C_2	8	C_{10}	35–50	(178)
C_{10}	4	C_{14}	38	(178)
C_6	8	C_{14}	44	(178)
C_{14}	2	C_{16}	30	(182)
C_{14}	4	C_{18}	28	(178)
C_{10}	8	C_{18}	18–20	(178)
C_2	16	C_{18}	25	(178)
C_{18}	2	C_{20}	25	(182)
	(ii) *Unsaturated acids*			
C_6	$HO_2C\cdot[CH_2]_3\cdot C{\equiv}C\cdot[CH_2]_3\cdot CO_2R$	$CH_3\cdot[CH_2]_7\cdot C{\equiv}C\cdot[CH_2]_3\cdot CO_2H$	25	(183)
C_5	$HO_2C\cdot[CH_2]_4\cdot C{\equiv}C\cdot[CH_2]_4\cdot CO_2R$	$CH_3\cdot[CH_2]_7\cdot C{\equiv}C\cdot[CH_2]_4\cdot CO_2H$	25	(183)
$CH_2{:}CH\cdot[CH_2]_8\cdot CO_2H$	4	$CH_2{:}CH\cdot[CH_2]_{12}\cdot CO_2H$	22	(179), (181)
$CH_3\cdot[CH_2]_5\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H$ (cis)	2	$CH_3\cdot[CH_2]_5\cdot CH{:}CH\cdot[CH_2]_9\cdot CO_2H$ (cis)	12	(118)

Table 1—(contd.)

*Monobasic acid**	*Dibasic acid† (half ester)* $HO_2C\cdot[CH_2]_n\cdot CO_2R$	*Product (after hydrolysis)*	*Yield, %*	*Ref.*
		(ii) *Unsaturated acids (contd.)*		
threo $CH_3\cdot[CH_2]_5\cdot CH(OH)\cdot CH(OH)\cdot[CH_2]_7\cdot CO_2H$	2	threo $CH_3\cdot[CH_2]_5\cdot CH(OH)\cdot CH(OH)\cdot[CH_2]_9\cdot CO_2H$ (thence trans acid, 42%)	23	(118)
$CH_3\cdot[CH_2]_7\cdot C{\equiv}C\cdot[CH_2]_4\cdot CO_2H$	3	$CH_3\cdot[CH_2]_7\cdot C{\equiv}C\cdot[CH_2]_7\cdot CO_2H$	6	(183)
$CH_3\cdot[CH_2]_7\cdot C{\equiv}C\cdot[CH_2]_3\cdot CO_2H$	4	$CH_3\cdot[CH_2]_7\cdot C{\equiv}C\cdot[CH_2]_7\cdot CO_2H$ (thence oleic acid, 74%)	24	(183)
C_8	$HO_2C\cdot[CH_2]_4\cdot C{\equiv}C\cdot[CH_2]_4\cdot CO_2R$	$CH_3\cdot[CH_2]_{10}\cdot C{\equiv}C\cdot[CH_2]_4\cdot CO_2H$ (thence cis ethylenic acid, 47%)	23	(180)
threo $CH_3\cdot[CH_2]_7\cdot CH(OH)\cdot CH(OH)\cdot[CH_2]_7\cdot CO_2H$	2	threo $CH_3\cdot[CH_2]_7\cdot CH(OH)\cdot CH(OH)\cdot[CH_2]_9\cdot CO_2H$	13	(182)
cis/trans $CH_3\cdot[CH_2]_7\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H$	4	cis/trans $CH_3\cdot[CH_2]_7\cdot CH{:}CH\cdot[CH_2]_{11}\cdot CO_2H$	*c.* 30	(181)
threo/erythro $CH_3\cdot[CH_2]_7\cdot CH(OH)\cdot CH(OH)\cdot[CH_2]_7\cdot CO_2H$	4	threo/erythro $CH_3\cdot[CH_2]_7\cdot CH(OH)\cdot CH(OH)\cdot[CH_2]_{11}\cdot CO_2H$ (thence ethylenic acids, 30–40%)	*c.* 30	(181)
$CH_3\cdot[CH_2]_7\cdot C{\equiv}C\cdot[CH_2]_7\cdot CO_2H$	4	$CH_3\cdot[CH_2]_7\cdot C{\equiv}C\cdot[CH_2]_{11}\cdot CO_2H$ (thence erucic acid, 88%)	28	(183)
cis/trans $CH_3\cdot[CH_2]_7\cdot CH{:}CH\cdot[CH_2]_7\cdot CO_2H$	6	cis/trans $CH_3\cdot[CH_2]_7\cdot CH{:}CH\cdot[CH_2]_{13}\cdot CO_2H$	30–35	(117)

* Saturated acids are indicated by chain length.
† Dibasic acids are indicated by the value of *n* where appropriate.

with the alkali metals, usually in liquid ammonia solution, to give alkali metal derivatives; acetylene itself forming a mono- or di-substituted derivative,

$$HC{:}CH \rightarrow HC{:}CNa \rightarrow NaC{:}CNa$$

$$R{\cdot}C{:}CH \rightarrow R{\cdot}C{:}CNa$$

These compounds react with carbon dioxide to give the corresponding carboxylic acid and condense with alkyl halides (or the more reactive sulphates, toluene-*p*-sulphonates, or methanesulphonates) or with carbonyl compounds; these reactions are illustrated by the following:

$$R{\cdot}C{:}CNa \xrightarrow{CO_2} R{\cdot}C{:}C{\cdot}CO_2H$$

$$HC{:}CNa \xrightarrow{RX} HC{:}C{\cdot}R \xrightarrow[(ii)\ R'X]{(i)\ Na} R'{\cdot}C{:}C{\cdot}R$$

$$HC{:}CH \nearrow \quad \searrow NaC{:}CNa \xrightarrow{2RX} R{\cdot}C{:}C{\cdot}R$$

$$R{\cdot}C{:}CNa \xrightarrow{R'_2CO} R{\cdot}C{:}C{\cdot}C(OH)R'_2$$

Of these, the reactions with the alkyl halides have been most widely used in the synthesis of aliphatic acids.

(*b*) Acetylene and ethynyl compounds also react with ethylmagnesium bromide to form the Grignard derivatives. Though similar to the alkali metal compounds they differ from them in some important respects especially in that acetylene forms directly a disubstituted compound and in that the Grignard compounds are almost completely unreactive towards saturated alkyl halides though the sulphates and sulphonates react normally as do allyl halides.

(*c*) GLASER[185] in 1870 reported that in the presence of air copper phenylacetylene readily couples with itself. This reaction is known to be applicable to many ethynyl compounds and, more important, to occur with two different ethynyl compounds so that the product of "crossed" coupling accompanies the two products of symmetrical coupling. The reaction is effected under very mild conditions (shaking at room-temperature with aqueous cuprous chloride-ammonium chloride solution in an atmosphere of oxygen) and proceeds in high yield.

$$2Ph{\cdot}C{:}CH \rightarrow Ph{\cdot}C{:}C{\cdot}C{:}C{\cdot}Ph$$

2. *The conversion of acetylenic intermediates to aliphatic acids*

Having obtained the acetylenic intermediate it is necessary to change it to the desired compound. In addition to using standard reactions such as those described in the previous section acetylenic compounds undergo some specific reactions of great usefulness, in particular stereospecific partial hydrogenation and bond migration.

(*a*) Triply bonded compounds are readily hydrogenated under catalytic conditions to the corresponding saturated compounds but the partial catalytic

hydrogenation (semi-hydrogenation) of such compounds to their ethylenic analogues, which also occurs smoothly under appropriate conditions, is of greater preparative interest, particularly as this reaction is stereospecific and yields almost entirely the cis isomer. This reaction was originally effected with a supported palladium catalyst but the partially poisoned catalyst of LINDLAR[186] is now usually preferred. This is a palladium—calcium-carbonate catalyst somewhat deactivated by treatment with lead acetate; the favoured solvent is petroleum ether containing some quinoline which is reported to further enhance selectivity. This catalyst may be safely used for semi-hydrogenation of one or more acetylenic bonds even when these are conjugated with ethylenic centres (e.g. reference 187)

$$—C{\vdots}C— \xrightarrow[\text{Na—NH}_3]{\text{yst}} \begin{cases} —CH{:}CH— \text{ (cis)} \\ —CH{:}CH— \text{ (trans)} \end{cases}$$

$$—CH(OH)\cdot C{\vdots}C— \xrightarrow{LiAlH_4} —CH(OH)\cdot CH{:}CH— \text{ (trans)}$$

Reduction by chemical processes, of which the most useful is a liquid ammonia solution of sodium or lithium, proceeds smoothly and leads entirely to the trans ethylenic compound.[188] Lithium aluminium hydride does not normally react with triple bonds unless adjacent to a hydroxyl group; they are then reduced to the trans ethylene.[189]

(*b*) It has recently been shown that an acetylenic bond may migrate as such or may be converted to an allene or conjugated diene when treated with alkali.[190] These changes are illustrated by the following example:

$$HO_2C\cdot CH_2\cdot C{\vdots}C\cdot CH_2\cdot CO_2H \xrightarrow{KOH} HO_2C\cdot \overset{trans}{CH{:}CH}\cdot \overset{trans}{CH{:}CH}\cdot CO_2H \xleftarrow{KOH} HO_2C\cdot C{\vdots}C\cdot CH_2\cdot CH_2\cdot CO_2H$$

$$(\cdot C{\vdots}C\cdot CH_2\cdot CH_2\cdot CH{:}CH\cdot CO_2H)_2 \xrightarrow{KOH} HO_2C\cdot [CH{:}CH]_6\cdot CO_2H \xleftarrow{KOH} (\cdot C{\vdots}C\cdot CH{:}CH\cdot CH_2\cdot CH_2\cdot CO_2H)_2$$

Corticrocin

$$HC{\vdots}C\cdot CH_2\cdot CO_2H \xrightarrow[40°]{K_2CO_3\,(20\%)} H_2C{:}C{:}CH\cdot CO_2H \,(92\%) \xrightarrow[90°]{K_2CO_3\,(20\%)} CH_3\cdot C{\vdots}C\cdot CO_2H \,(60\%)$$

These facile changes have found their greatest application in the synthesis of polyenes (such as corticrocin) which are outside the scope of this article.[191, 192]

3. *Syntheses involving the use of organometallic acetylenic compounds*

The simplest application of acetylenic compounds to the synthesis of aliphatic acids is the carbonation of the alkali metal or Grignard compounds. This leads

to the $\alpha\beta$-acetylenic acids (carbon dioxide) or esters (ethyl carbonate or ethyl chloroformate) and hence to the $\alpha\beta$-ethylenic analogues.[193–197]

$$R\cdot C{\vdots}CH \rightarrow \underset{(M = Na \text{ or } MgX)}{R\cdot C{\vdots}C\cdot M} \rightarrow \underset{(R' = H \text{ or } Et)}{R\cdot C{\vdots}C\cdot CO_2R'} \rightarrow R\cdot CH{:}CH\cdot CO_2R'$$

In 1928 behenolic acid was obtained by condensing a sodium acetylide with

$$CH_3\cdot[CH_2]_7\cdot C{\vdots}CNa + Br[CH_2]_{11}\cdot CO_2Me \rightarrow CH_3\cdot[CH_2]_7\cdot C{\vdots}C\cdot[CH_2]_{11}\cdot CO_2H$$

an ω-bromo ester.[198] Ten years later tetradec-5-ynoic acid was prepared by the condensation of dec-1-yne with 3-chloropropyl toluene-*p*-sulphonate in the presence of sodamide, the condensation product being subsequently converted into the acid via the nitrile.[199]

$$CH_3\cdot[CH_2]_7\cdot C{\vdots}CH + TsO\cdot[CH_2]_3Cl \rightarrow CH_3\cdot[CH_2]_7\cdot C{\vdots}C\cdot[CH_2]_3Cl \rightarrow CH_3\cdot[CH_2]_7\cdot C{\vdots}C\cdot[CH_2]_3\cdot CO_2H$$

Another ten years elapsed before the general usefulness of this reaction was demonstrated by STRONG and his co-workers,[200–202] who showed that ethynyl compounds (XXVII) interact, as their sodium derivatives or Grignard complexes, with $\alpha\omega$-dihalogeno compounds (XXVIII). The resulting acetylenic compound (XXIX) is readily converted into an acid with one more carbon atom via the cyanide or to an acid with two more carbon atoms by malonation and then by semi-hydrogenation to the cis ethylenic acid (XXX). This is of particular importance since it permits the synthesis of the naturally occurring cis acids.

$$\underset{(XXVII)}{CH_3\cdot[CH_2]_m\cdot C{\vdots}CH} + \underset{(XXVIII)}{I[CH_2]_nCl} \rightarrow \underset{(XXIX)}{CH_3\cdot[CH_2]_m\cdot C{\vdots}C\cdot[CH_2]_nCl} \rightarrow \underset{(XXX)}{CH_3\cdot[CH_2]_m\cdot CH{:}CH\cdot[CH_2]_{n(+1)}\cdot CO_2H}$$

This method has been used to prepare a wide range of acids[150, 203, 208] and is illustrated in the examples given below. For practical reasons the lithium derivative is sometimes preferred to the sodium compound.

(*a*) *Synthesis of octadec-trans-*11*-enoic (vaccenic) acid.*[201]

$$CH_3\cdot[CH_2]_5\cdot C{\vdots}CH + I[CH_2]_9Cl \xrightarrow{Na—NH_3} CH_3\cdot[CH_2]_5\cdot C{\vdots}C\cdot[CH_2]_9Cl$$

$$\xrightarrow[\text{(ii) KOH}]{\text{(i) NaCN}} CH_3\cdot[CH_2]_5\cdot C{\vdots}C\cdot[CH_2]_9\cdot CO_2H \xrightarrow{H_2—Ni}$$

$$CH_3\cdot[CH_2]_5\cdot \overset{cis}{CH{:}CH}\cdot[CH_2]_9\cdot CO_2H \xrightarrow{Se} CH_3\cdot[CH_2]_5\cdot \overset{trans}{CH{:}CH}\cdot[CH_2]_9\cdot CO_2H$$

(*b*) *Synthesis of* (±) *ricinoleic acid.*[209, 210]

$$CH_3\cdot[CH_2]_5\cdot CHO \xrightarrow[Zn]{BrCH_2\cdot C\vdots CH} CH_3\cdot[CH_2]_5\cdot CH(OH)\cdot CH_2\cdot C\vdots CH + CH_3\cdot[CH_2]_5\cdot CH(OH)\cdot CH{:}C{:}CH_2$$

$$\downarrow \text{(i) Dihydropyran-H}^+ \text{; (ii) } NaNH_2$$

$$CH_3\cdot[CH_2]_5\cdot CH(OPy)\cdot CH_2\cdot C\vdots CNa \xrightarrow{I[CH_2]_6Cl}$$

(Py = tetrahydro-2-pyranyl)

$$CH_3\cdot[CH_2]_5\cdot CH(OPy)\cdot CH_2\cdot C\vdots C\cdot[CH_2]_6Cl \xrightarrow[(ii)\ CH_2(CO_2Et)_2,\ etc.]{(i)\ NaI}$$

$$CH_3\cdot[CH_2]_5\cdot CH(OH)\cdot CH_2\cdot C\vdots C\cdot[CH_2]_7\cdot CO_2H \xrightarrow{H_2\text{-Lindlar}}$$

$$CH_3\cdot[CH_2]_5\cdot CH(OH)\cdot CH_2\cdot \overset{cis}{CH{:}CH}\cdot[CH_2]_7\cdot CO_2H$$

(*c*) *Synthesis of methyl trideca-trans-*3*:cis-*5*- and trans-*3*:trans-*5*-dienoates.*[211]

$$CH_3\cdot[CH_2]_6\cdot C\vdots CH \xrightarrow[(ii)\ CH_2{:}CH\cdot CHO]{(i)\ EtMgBr} CH_3\cdot[CH_2]_6\cdot C\vdots C\cdot CH(OH)\cdot CH{:}CH_2$$

$$\xrightarrow[(ii)\ rearrangement]{(i)\ PBr_3—C_5H_5N} CH_3\cdot[CH_2]_6\cdot C\vdots C\cdot CH{:}CH\cdot CH_2Br \xrightarrow[(ii)\ MeOH—HCl]{(i)\ Cu_2(CN)_2—NaI}$$

$$CH_3\cdot[CH_2]_6\cdot C\vdots C\cdot CH{:}CH\cdot CH_2\cdot CO_2Me \xrightarrow{Lindlar}$$

$$CH_3\cdot[CH_2]_6\cdot \overset{cis}{CH{:}CH}\cdot \overset{trans}{CH{:}CH}\cdot CH_2\cdot CO_2Me \xrightarrow{I_2}$$

$$CH_3\cdot[CH_2]_6\cdot \overset{trans}{CH{:}CH}\cdot \overset{trans}{CH{:}CH}\cdot CH_2\cdot CO_2Me$$

(*d*) Some excellent examples of the control available by these methods are found in the various methods used to synthesize deca-2:4-dienoic,[212] deca-2:6-dienoic,[196, 213] and dodeca-2:8-dienoic acids,[195, 197, 214] each of which has been synthesized in its four possible stereochemical forms. The synthesis of the four deca-2:4-dienoic acids is given below; the trans-2:cis-4 isomer was found to be identical with the decadienoic acid present in stillingia oil.

(i) Deca-trans-2:trans-4-dienoic acid.

$$CH_3\cdot[CH_2]_4\cdot C\vdots CH \xrightarrow[(ii)\ CH(OEt)_3]{(i)\ MeMgBr} CH_3\cdot[CH_2]_4\cdot C\vdots C\cdot CH(OEt)_2 \xrightarrow{Pd—H_2}$$

$$CH_3\cdot[CH_2]_4\cdot \overset{cis}{CH{:}CH}\cdot CH(OEt)_2 \xrightarrow{H^+} CH_3\cdot[CH_2]_4\cdot \overset{trans}{CH{:}CH}\cdot CHO$$

$$\xrightarrow{Doebner\ reaction} CH_3\cdot[CH_2]_4\cdot \overset{trans}{CH{:}CH}\cdot \overset{trans}{CH{:}CH}\cdot CO_2H$$

(ii) Deca-trans-2:cis-4-dienoic acid.

$$CH_3\cdot[CH_2]_4\cdot C\vdots C\cdot CHO \xrightarrow{Reformatsky\ reaction}$$

$$CH_3\cdot[CH_2]_4\cdot C\vdots C\cdot CH(OH)\cdot CH_2\cdot CO_2Et \xrightarrow[(ii)\ NaOH]{(i)\ POCl_3—C_5H_5N}$$

$$CH_3\cdot[CH_2]_4\cdot C\vdots C\cdot CH{:}CH\cdot CO_2H \xrightarrow[(ii)\ Lindlar]{(i)\ CH_2N_2}$$

$$CH_3\cdot[CH_2]_4\cdot \overset{cis}{CH{:}CH}\cdot \overset{trans}{CH{:}CH}\cdot CO_2Me$$

(iii) Deca-cis-2:trans-4-dienoic acid.

$$CH_3\cdot[CH_2]_4\cdot CHO \xrightarrow[Zn]{HC{\equiv}C\cdot CH_2Br} CH_3\cdot[CH_2]_4\cdot CH(OH)\cdot CH_2\cdot C{\equiv}CH$$

$$\xrightarrow[H^+]{\text{dihydropyran}} CH_3\cdot[CH_2]_4\cdot CH(OPy)\cdot CH_2\cdot C{\equiv}CH \xrightarrow[(ii)\ CO_2]{(i)\ EtMgBr}$$

(Py = tetrahydro-2-pyranyl)

$$CH_3\cdot[CH_2]_4\cdot CH(OPy)\cdot CH_2\cdot C{\equiv}C\cdot CO_2H \xrightarrow[Lindlar]{MeOH-H^+}$$

$$CH_3\cdot[CH_2]_4\cdot CH(OH)\cdot CH_2\cdot \overset{cis}{CH{:}CH}\cdot CO_2Me \xrightarrow{HCl}$$

$$CH_3\cdot[CH_2]_4\cdot CH\cdot CH_2\cdot \overset{cis}{CH{:}CH}\cdot C{:}O \xrightarrow{NaOMe-MeOH}$$

$$\underset{\text{O}}{\text{|}}$$

$$CH_3\cdot[CH_2]_4\cdot \overset{trans}{CH{:}CH}\cdot \overset{cis}{CH{:}CH}\cdot CO_2H$$

(iv) Deca-cis-2:cis-4-dienoic acid.

$$ClCH_2\cdot C{\equiv}C\cdot CH_2Cl \xrightarrow{3NaNH_2} NaC{\equiv}C\cdot C{\equiv}CH \xrightarrow{CH_3\cdot[CH_2]_4Br}$$

$$CH_3\cdot[CH_2]_4\cdot C{\equiv}C\cdot C{\equiv}CH \xrightarrow[(ii)\ CH_2N_2]{(i)\ EtMgBr-CO_2} CH_3\cdot[CH_2]_4\cdot C{\equiv}C\cdot C{\equiv}C\cdot CO_2Me$$

$$\xrightarrow{Lindlar} CH_3\cdot[CH_2]_4\cdot \overset{cis}{CH{:}CH}\cdot \overset{cis}{CH{:}CH}\cdot CO_2Me$$

(*e*) One of the early successes of this method was the preparation of linoleic acid achieved independently by three groups of investigators.[215–217]*

(i) Raphael and Sondheimer (1950).[215]

$$CH_3\cdot[CH_2]_4\cdot C{\equiv}CH \qquad Cl[CH_2]_6Cl$$

$$\downarrow \text{(i) EtMgBr, (ii) } CH_2O \qquad \downarrow \text{(i) NaI, (ii) } HC{\equiv}CNa$$

$$CH_3\cdot[CH_2]_4\cdot C{\equiv}C\cdot CH_2OH$$

$$\downarrow CH_3\cdot SO_2Cl$$

$$CH_3\cdot[CH_2]_4\cdot C{\equiv}C\cdot CH_2OMs \qquad HC{\equiv}C\cdot[CH_2]_6Cl$$

(Ms = Mesyl, CH_3SO_2)

$$\downarrow EtMgBr$$

$$CH_3\cdot[CH_2]_4\cdot C{\equiv}C\cdot CH_2\cdot C{\equiv}C\cdot[CH_2]_6Cl$$

$$\downarrow \text{(i) NaI, (ii) malonation, (iii) } H_2\text{—Pd}$$

$$CH_3\cdot[CH_2]_4\cdot \overset{cis}{CH{:}CH}\cdot CH_2\cdot \overset{cis}{CH{:}CH}\cdot[CH_2]_7\cdot CO_2H$$

* Syntheses of elaeostearic acid[217a] and linolenic acid[217b] have been reported.

(ii) GENSLER and THOMAS (1951).[216]

$$CH_3\cdot[CH_2]_4\cdot C{\vdots}C\cdot CH_2Br + HC{\vdots}C\cdot[CH_2]_6Cl \xrightarrow[Cu_2Cl_2]{EtMgBr}$$

$$CH_3\cdot[CH_2]_4\cdot C{\vdots}C\cdot CH_2\cdot C{\vdots}C\cdot[CH_2]_6Cl \xrightarrow[(ii)\ Malonation]{(i)\ NaI}$$

$$CH_3\cdot[CH_2]_4\cdot C{\vdots}C\cdot CH_2\cdot C{\vdots}C\cdot[CH_2]_7\cdot CO_2H \xrightarrow{H_2}$$

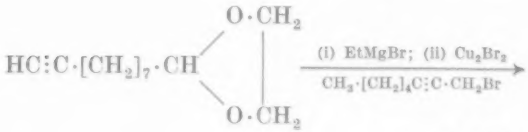
$$CH\cdot[CH_2]_4\cdot \overset{cis}{CH{:}CH}\cdot CH_2\cdot \overset{cis}{CH{:}CH}\cdot[CH_2]_7\cdot CO_2H$$

(iii) WALBORSKY, DAVIES, and HOWTON (1951).[217]

$$HC{\vdots}C\cdot[CH_2]_6\cdot C{\vdots}CH \xrightarrow{CH_3\cdot COSH}$$

$$HC{\vdots}C\cdot[CH_2]_6\cdot CH{:}CHS\cdot CO\cdot CH_3 \xrightarrow{NH_2OH}$$

$$HC{\vdots}C\cdot[CH_2]_6\cdot CH_2\cdot CH{:}NOH \xrightarrow[H^+]{(CH_2OH)_2}$$



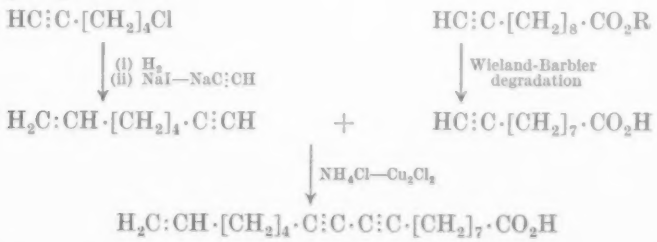
$$CH_3\cdot[CH_2]_4\cdot C{\vdots}C\cdot CH_2\cdot C{\vdots}C\cdot[CH_2]_7\cdot CO_2H \xrightarrow{H_2—Ni}$$

$$CH_3\cdot[CH_2]_4\cdot \overset{cis}{CH{:}CH}\cdot CH_2\cdot \overset{cis}{CH{:}CH}\cdot[CH_2]_7\cdot CO_2H$$

4. *Syntheses involving oxidative coupling*

Whilst the acetylenic organometallic compounds have given satisfactorily many mono- and di-ethenoid acids, the more highly unsaturated compounds are better obtained by "crossed" oxidative coupling of two ethynyl compounds. This method is illustrated by the synthesis of erythrogenic acid[87] and of methyl deca-trans-2-en-4:6:8-triynoate.[218]

(*a*) *Erythrogenic acid.*



(*b*) *Methyl deca-trans-2-ene-4:6:8-triynoate.*

$$CH_3{\cdot}C{\equiv}C{\cdot}C{\equiv}CH + HC{\equiv}C{\cdot}CH{:}CH{\cdot}CO_2Me \xrightarrow[Cu_2Cl_2]{NH_4Cl} CH_3{\cdot}C{\equiv}C{\cdot}C{\equiv}C{\cdot}C{\equiv}C{\cdot}CH{:}CH{\cdot}CO_2Me$$

G. Other methods

KUHN[219] has reported that acetaldehyde or crotonaldehyde undergoes condensation in presence of piperidine acetate, yielding polyene aldehydes, $R{\cdot}[CH{:}CH]_nCHO$. These may be reduced to saturated alcohols and then oxidized to the acids, or condensed with malonic acid before hydrogenation. Some saturated and unsaturated acids have been so prepared.[220–223]

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2

THE NATURALLY OCCURRING ACETYLENIC ACIDS

Edwin M. Meade

CONTENTS

		Page
I.	INTRODUCTION	47
II.	HISTORY	47
III.	OCCURRENCE OF ACETYLENIC ACIDS	48
IV.	THE GLYCERIDE ACETYLENES	48
	A. Tariric acid	48
	B. Ximenynic or santalbic acid	49
	C. Ximenynolic acid	49
	D. Isanic or erythrogenic acid	49
	E. Bolekic acid	50
	F. Isanolic acid	50
V.	THE NON-GLYCERIDE ACETYLENES	51
	A. The C_8 group	51
	1. Diatetryne	51
	2. Clitocybin	51
	3. Agrocybin	51
	B. The C_{10} group	51
	1. Isano decenediyne	51
	2. Lachnophyllum ester	51
	3. Dihydromatricaria ester	51
	4. Matricaria ester	52
	5. Matricarianol	52
	6. Dehydromatricaria ester	52
	C. The C_{11} group	52
	1. Nemotinic acid	52
	2. Nemotin	53
	3. Odyssin	53
	D. The C_{12} group	54
	1. Artemisia ketone	54
	2. Centaur X	54
	E. The C_{13} group	54
	1. Mycomycin	54
	2. Isomycomycin	54
	3. Triene-triynyl acetate from *Carlina vulgaris*	54
	4. The coreopsis poly-ynes	54

F. The C_{14} group 55
1. Artemisia ketone 55
2. Anacycline 55
3. Anacycline conjugated enediyne 55
G. The C_{17} group (*Oenanthe* and *Cicuta* poly-ynes) 55

VI. ISOLATION AND PHYSICAL PROPERTIES 56
A. Properties used in separation 56
B. Infra-red spectra 57
C. Ultra-violet spectra 57

VII. CHEMICAL PROPERTIES 57

VIII. PHYSIOLOGICAL PROPERTIES 59

IX. SYNTHESIS 59

X. CONCLUSION 60

REFERENCES 60

2

THE NATURALLY OCCURRING ACETYLENIC ACIDS

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I. Introduction

The first acetylenic compound recognized as occurring in nature was the eighteen carbon atom tariric acid, found by Arnaud in a fatty oil in 1892, and as an intriguing anomaly it is still the only known simple natural acetylene, all the twenty or so others being conjugated with other unsaturated centres. Compounds up to the pentayne stage have been isolated, but from triyne onwards they become increasingly unstable, at least three naturally occurring explosives being in the range. The precise techniques of laboratory acetylenic synthesis come to a halt at present at the triyne stage in mono-basic acids, but they have led to the position where the constitution of a new acetylenic product is now often proved by synthesis within a year of its first isolation.

As the study of the natural acetylenic acids provides the smaller context, and is more in line with the general terms of reference of this series, it will be the main theme of this review, and acetylenic synthesis [which is excellently reported elsewhere (Raphael, 1955)] will be mentioned only within its relevance to the main topic. The realm of lipid chemistry is interpreted broadly however, and non-glyceride sources of natural acetylenic acids are included in the discussion.

II. History

The partial synthesis of acetylenic fatty acids preceded the recognition of their natural occurrence by a quarter of a century. Overbeck made stearolic acid from oleic and elaidic dibromides in 1866, and his use of alcoholic potash was repeated to yield palmitolic, behenolic, and ricinstearolic acids in the following year. Arnaud in 1892 provided the next of the widely spaced early landmarks, isolating a new acid from the seed fat of *Picramnia tariri*, which he recognized immediately as an iso-stearolic acid, and established later (1900) as the 6:7 individual. The first complete synthesis did not come until over sixty years after Overbeck's work, Simonsen (1928) obtaining the C_{22} behenolic acid, apparently in small yield, from the high temperature condensation of sodiodecyne with methyl 12-bromdodecanoate, the decyne moiety being prepared in liquid ammonia as a reaction medium, which curiously was abandoned for the final condensation.

Forty-three years after Arnaud's tariric acid, Wiljams, Smirnov, and Goljmov (1935) isolated and proved the structure of the C_{10} enediyne

"lachnophyllum ester", and two years later STEGER and VAN LOON (1937) found the third naturally occurring aliphatic acetylene in the oil from the nut of the "boleko" or "isano" tree (*Onguekoa sp.*), their isanic acid being a C_{18} acid with mid-chain conjugated diacetylenic unsaturation and a terminal double bond. No further progress occurred until the post-war period, when work expanded rapidly, the acetylenic syntheses becoming standard for fatty acid work, a new source of acetylenic glycerides being found in *Ximenia sp.* fruits, and the remarkable field of the polyethynoid higher plant and fungus lipids being opened wide by the Anchel, E. R. H. Jones and Sørensen groups (see below), culminating in a conjugated enepentayne, which is the first natural colouring matter known to owe its colour to a poly-yne system.

In a field of such accelerating development, the future is especially obscure, but it is unlikely that poly-ynes can be so widespread in nature without many so far unsuspected appearances of mono-acetylenes.

III. Occurrence of Acetylenic Acids

Only three sources of acetylenic glycerides are known as yet, *Picramnia sp.* for tariric acid, the closely related *Santalum* and *Ximenia sp.* for ximenynic acid, and *Onguekoa sp.* for the isanic/bolekic acid group, only the fruit kernels having been examined in each case. "Single chain" compounds such as esters and amides are much more widely distributed, especially in the Compositae, and are distributed throughout the plant, though the root system seems to be the most dependable source, and crude steam distillates of common composite weed plants are in some cases poly-ynes of quite high purity. In *Coreopsis* one individual is present throughout the plant, but quite different ones accumulate specifically in the roots, the leaves, and the flowers. Certain Basidiomycetes contain acetylenic materials, and here extreme instability complicates the isolation of pure individuals, some of which are trebly unique in being explosive, in containing terminal acetylenic groups, and also in having allenic structures. The possibility of the optical resolution of disubstituted allenes was a classical problem early in this century, and we now have the ironic knowledge that nature solved the problem millions of years ago, and left the evidence around us in the form of some of the common fungi.

Nothing is known of the biogenesis of acetylenes, which are much higher energy compounds than the olefins, where the biogenetic routes are almost equally obscure, but the ability to effect particular acetylenic syntheses seems to be one of the more finely distinguishing points in species and sub-species specificity, and the SØRENSENS have already indicated its use as an aid to botanical classification.

IV. The Glyceride Acetylenes

The few known members of this group are all straight chain C_{18} individuals.

A. "Tariric" acid, 6-octadecynoic acid

$$CH_3(CH_2)_{10}—C{\equiv}C—(CH_2)_4—COOH, \text{ M.pt. } 50{\cdot}5°C$$

This occurs in the fruit kernel oil of various *Picramnia sp.* (ARNAUD, 1892; GRIMME, 1910, 1912), and above all in that of *P.sow* (STEGER and VAN LOON, 1933) which is almost pure tri-taririn, with 95 per cent of tariric acid in its fatty acid composition. The acid forms a di-iodide M.pt. 48·5°C, a dibromide M.pt. 32°C, a tetrabromide M.pt. 125°C, and hydrogenates to petroselenic acid and then stearic acid.

*B. "Ximenynic" or "santalbic" acid** 11-(*trans*)-*octadecene*-9-*ynoic acid*

$$CH_3(CH_2)_5—CH=CH—C\equiv C—(CH_2)_7—COOH, \text{ M.pt. } 39°C$$

This occurs to the extent of 20–25 per cent in the seed oils of *Ximenia sp.* (LIGTHELM, 1952), and 40–50 per cent in *Santalum sp.* oils (GUNSTONE, 1954; HATT, 1954), but in the santalum oils it is the only unusual acid, whereas in ximenia oils it is associated with 3–12 per cent each of the C_{24}, C_{26}, C_{28}, and C_{30} olefinic acids, all of the series $C_8H_{17}—CH=CH—(CH_2)_n—COOH$. It forms a methyl ester b.p. 169°C/1 mm, some 10°C above the usual C_{18} range, a bromphenacyl ester M.pt. 53–54°C, and amide M.pt. 105°C. On performic acid oxidation followed by hydrolysis it gives an 11:12-dihydroxystearolic acid of M.pt. 88–89°C, and hydrogenates smoothly to stearic acid with no recorded major intermediate stage. It has been synthesized from ricinstearolic acid via chlorstearolic acid (GRIGOR, 1954).

C. "Ximenynolic acid", 8-hydroxy-ximenynic acid, 8-hydroxy-11-(*trans*)-*octadecen*-9-*ynoic acid*

$$CH_3—(CH_2)_5—CH=CH—C\equiv C—CHOH—(CH_2)_6COOH$$

This occurs as a minor component acid (3–4 per cent) in *Ximenia caffra* oil (LIGTHELM, 1954). Isolated by solvent partition, it was characterized by its eneyne u.v. absorption, oxidation, and hydrogenation to 8-hydroxy-stearic acid. Lithium aluminium hydride reduced the ester to a trans-trans conjugated dienediol, a reductive behaviour restricted to α-hydroxy acetylenes.

D. "Isanic" or "erythrogenic" acid,† 17-*octadecene*-9:11-*diynoic acid*

$$CH_2=CH—(CH_2)_4—C\equiv C—C\equiv C—(CH_2)_7—COOH, \text{ M.pt. } 40°C$$

This acid occurs to some 40 per cent in the nut oil of the "isano" or "boleko" tree, *Onguekoa Gore*, an oil commercially available from the Belgian Congo. Like many diacetylenes, the acid reddens on storage, hence its second name (CASTILLE, 1939). This seems to be a solid state phenomenon only, and the red pigment produced is insoluble in organic solvents even when strongly alkaline. Isanic acid gives a zero diene value with maleic anhydride and an iodine value corresponding to a three-fold addition (STEGER and VAN LOON, 1940), is oxidized by permanganate selectively to 17:18-dihydroxyoctadeca-9:11-diynoic acid

* The former seems to be the better established.

† The former name is better established, and has precedence on priority (STEGER and VAN LOON, 1937) assonance, and relevance.

of M.pt. 92°C (CASTILLE, 1939), and adds hydrogen preferentially to the terminal double bond (CASTILLE, 1941). It has been synthesized by the oxidative coupling of ω-octenyne and ω-decynoic acid (WEEDON, 1953). It forms a methyl ester b.p. 175°C/1 mm which does not redden on storage, D_{20}^{20} 0·9350, N_D^{20} 1·4939, and from the ester a β-hydroxyethylamide, M.pt. 67°C, which rapidly turns blue in the air (MEADE, 1950). The natural acid has molecular extinction maximum coefficients of 510/2390 Å and 620/2270 Å, corrected for a content of 2 per cent of the conjugated enediyne bolekic acid (MEADE, 1950): the synthetic acid shows values some 20 per cent lower, but both ethanolamides show identical values of 655/2270 Å (WEEDON, 1953), indicating the value of these easily formed derivatives for purification. The acid and methyl ester both form urea complexes of low stability (MEADE, 1951).

E. "Bolekic acid", conjugated mid-chain ene(cis)-diyne octadecanoic acid, M.pt. 17°C

This acid was isolated by its inability to form a urea complex at — 6°C, after removal of all hydroxyacids by phthalation (MEADE, 1951). It takes up 4·9 molecules of hydrogen to form stearic acid, shows cis-unsaturation by infra-red absorption (with signs of ω-unsaturation indicating some inhomogeneity), and typical conjugated ene-diyne ultra-violet absorption, with molecular extinction maxima of 18,600/2840 Å and 23,300/2680 Å (MEADE, 1951). It gives a methyl ester D_{20}^{20} 0·9413, N_D^{20} 1·5179, and thence a β-hydroxyethylamide M.pt. 47–48°C, which is fairly stable in air.

F. "Isanolic acid"

This is clearly inhomogeneous. It is a mixture of 8-hydroxy C_{18} acids as shown by hydrogenation to 8-hydroxystearic acid (RILEY, 1951). A pure hydroxy fraction, isolated by phthalation of boleko oil methyl esters, separation from neutral material, dephthalation by alkaline methanolysis, and finally molecular distillation, showed both diyne and enediyne conjugation to be present, and the oil to contain some 35 per cent of hydroxy-isanic and 5 per cent of hydroxy-bolekic type acids (MEADE, 1952). There is no room between C_8 and the carboxyl group for an ene-diyne system not conjugated with carboxyl, and this together with oxidation studies, though done on a material of only 40 per cent hydroxy acid content (SEHER, 1954) confirm these constitutions. Further circumstantial evidence can be adduced from the fact that CASTILLE (1939) found a decene-ω-diyne in the oil—either heat or alkaline refining could produce this from 8-hydroxy-isanic acid, but from no other isomer, by a reversed Nef reaction:

$$CH_2{=}CH{-}(CH_2)_4{-}C{\equiv}C{-}C{\equiv}C{-}CHOH{-}(CH_2)_6{-}COOH \rightarrow$$
$$CH_2{=}CH{-}(CH_2)_4{-}C{\equiv}C{-}C{\equiv}CH + CHO{-}(CH_2)_6{-}COOH$$

CASTILLE's hydrocarbon gave an argento derivative, proving the ω-acetylene, and the production of such a hydrocarbon at well above its spontaneous

ignition point may also explain the "explosions" recorded on cooking isano oil at varnish making temperatures.

V. The Non-Glyceride Acetylenes

A. The C_8 *group*

1. "*Diatetryne* 1". "Diatetryne Nitrile", 7-Cyano-2-heptene-4:6-diynoic acid, CN—C≡C—C≡C—CH=CH—COOH is an antibiotic found by Anchel (1953) elaborated by the fungus *Clitocybe diatetra*. It shows typical triyne absorption in the ultra-violet region, due to the nitrile-diyne conjugation, and this and its interconvertion with the better characterized clitocybin prove its constitution.

2. "*Clitocybin*". "Diatetryne 2", "Diatetryne Amide" (the first name seems to be the established one) 7-Carboxamido-2-heptene-4:6-diynoic acid, $CONH_2$—C≡C—C≡C—CH=CH—COOH, explodes at 198°C, accompanies "Diatetryne 1" in *Clitocybe*, has been obtained from it by partial hydration, and reconverted into it in small yield by dehydration (Anchel, 1953). It is 10^4 times less active as an antibiotic. Its structure has been proved by synthesis (Jones, 1954) supported by its pK value of 3·5, which corresponds to a $\beta\gamma$-ynoic acid, and not to the stronger $\alpha\beta$-acids, which show a pK range of 1·7 to 2·7.

3. "*Agrocybin*", 7-Carboxamido-6-heptene-4:6-diyne-1-ol,

$$CONH_2—C≡C—C≡C—CH=CH—CH_2OH,$$

explodes at 145°C, was found by Anchel (1953) in the fungus *Agrocybe dura*. It is unstable, turning black in one day at room temperature.

B. The C_{10} *group*

1. *Isano decenediyne*. This is probably an artefact, v. isanolic acid (IV F).

The remarkable close-knit C_{10} group of dehydrocapric acid derivatives is still under active investigation, and is perhaps specially unusual for the specificity of occurrence of different geometric forms. Less remarkable, but pleasantly noteworthy in this group, is the very close international co-operation between the Anchel (U.S.A.), Jones (U.K.), and Sørensen (Norway) research schools.

2. "*Lachnophyllum ester*".

$$CH_3—CH_2—CH_2—C≡C—C≡C—CH=CH—COOCH_3,$$

cis form M.pt. 33°C, trans 19°C. The cis-form was isolated and its structure proved by Wiljams (1935), and was then shown to occur widely in the Compositae by Sørensen (1950) though the common Norwegian wild daisy, *Bellis perennis*, contains the trans form (Sørensen, 1954). The trans form was synthesized in 1950 (Sørensen) and the cis form in 1956 (Jones).

3. *Dihydromatricaria ester*.

$$CH_3—CH=CH—C≡C—C≡C—CH_2—CH_2—COOCH_3$$

8-cis form, M.pt. 95·5°C isomeric with 2 above, is a minor component in the essential oil of scentless mayweed, *Matricaria inodorata* (SØRENSEN, 1952).

4. *Matricaria ester.*

$$CH_3—CH=CH—C\equiv C—C\equiv C—CH=CH—COOCH_3$$

cis-cis form M.pt. 35°C, trans-trans M.pt. 63°C, 2-cis-8-trans form, M.pt. 2°C. The cis-cis form is the major component in scentless mayweed (SØRENSEN, 1941) and has been synthesized (JONES, 1956): the other two forms were also synthesized earlier and the 2-cis-8-trans form found as a minor component in mayweed (SØRENSEN, 1952) and the trans-trans form in the fungus *Polyporus anthracophilus* (JONES, 1955).

5. *Matricarianol.* $CH_3—CH=CH—C\equiv C—C\equiv C—CH=CH—CH_2OH$, trans-trans form, M.pt. 107–108°C. This is present, probably as its acetate, in the essential oil of some *Asterae*, and occurs as the trans-trans form (SØRENSEN, 1954). The free trans-trans alcohol is present in the fungus *Polyporus anthracophilus* (JONES, 1955).*

6. *Dehydromatricaria ester.*

$$CH_3—C\equiv C—C\equiv C—C\equiv C—CH=CH_2—COOCH_3,$$

cis-form, M.pt. 114°C, trans M.pt. 105°C. The high melting cis-triyne crystallizes out spontaneously from the essential oil of *Artemisia vulgaris*, a behaviour noted as far back as 1826 (BRETZ). Sørensen showed its constitution in 1950, and Jones synthesized it in 1956. The faint yellow colour of the natural material perhaps arises from contamination with "Composit-Cumulene Ester I" (SØRENSEN, 1950) a remarkable non-acetylene of the same C_{10} series, probably $CH_3(CH_2)_5—CH=C=C=CHCOOCH_3$.

The trans form occurs in the roots of *M.inodorata* (SØRENSEN, 1954; BOHLMANN, 1956).

C. The C_{11} *group*

1. *"Nemotinic acid".*

$$CH\equiv C—C\equiv C—CH=C=CH—\underset{\displaystyle OH}{\underset{|}{CH}}—CH_2—CH_2—COOH$$

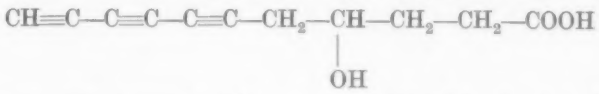
$$[\alpha_D^{17}] + 320°, \text{ pK } 4{\cdot}80$$

This antibiotic was isolated by ANCHEL (1950) from the fungi *Poria tenuis* and *Poria corticola*, accompanied by the corresponding lactone "nemotin", and the constitutions were proved by the E. R. H. JONES school (1955) and some earlier misconceptions about their alkaline rearrangement products fully cleared up (JONES, 1956).

Nemotinic acid is extremely unstable, except in dilute solution, is rearranged by alkali to iso-nemotinic acid, but is remarkably stable to acid, 8 per cent sulphuric acid in dioxane for four days at room temperature lactonizing it to nemotin without rearrangement in 60 per cent yield (JONES, 1955). In contrast

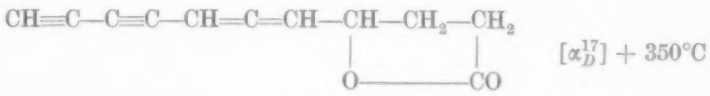
* Deca-2:8-diene-4:6-diyn-1:10-dioic acid was also found in the culture medium.

to the other natural ω-acetylene mycomycin, the alkaline rearrangements of the nemotins do not involve the terminal diyne system, iso-nemotinic acid being



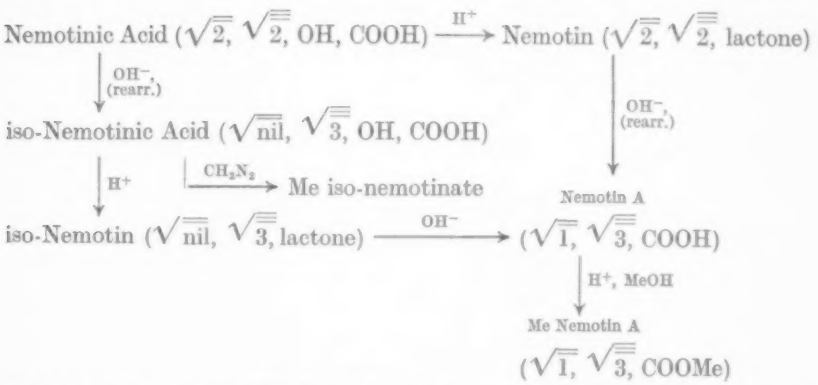
This weakly optically active ($[\alpha]_D^{20} = -3°$) acid is fairly stable to further alkaline treatment, and once again is acid stable, being lactonized to an iso-nemotin. This new lactone shows a different form of alkali instability, being saponified with water elimination to give nemotin A in 40 per cent yield.

2. "*Nemotin*".



This lactone accompanies nemotinic acid, forms the expected argento compound, and is rearranged by alkali (Anchel, 1950). This rearrangement is extremely facile (half-life 23 min. at *p*H 8·4), being some hundredfold faster than for nemotinic acid, and is accompanied by elimination of water, yielding nemotin A of the following constitution (Jones, 1956)

The relationships within this group as corrected by this latest work are as follows:



With all due gratitude for the benefits of instrumental analysis, it is unfortunate that the presence of the terminal acetylene in these two new methyl esters and the new lactone should have been proved only by their infra-red spectra, when the additional diagnostic chemical test of argento compound formation is so easily available: this "flight from chemistry" is particularly surprising in work from a school which has developed this test quantitatively (Whiting, 1953; cf. Raphael, 1955, p. 207).

3. "*Odyssin*", "*Odyssic acid*". These accompany the nemotins (Jones, 1955) and are described so far only as being poly-ynes.

D. The C_{12} *group*

1. *Artemisia ketone.* This is in the literature (SØRENSEN, 1950) as a C_{12} compound, but is now known to be C_{14} (see below).

2. *Centaur X.* This is obtained from *Centauria sp.*, and is probably inhomogeneous (SØRENSEN, 1954; LØFGREN, 1952) and probably contains the symmetrical diene-triyne hydrocarbon

$$CH_3—CH=CH—C\equiv C—C\equiv C—C\equiv C—CH=CH—CH_3$$

E. The C_{13} *group*

1. *"Mycomycin".*

$$CH\equiv C—C\equiv C—CH=C=CH—CH=CH—CH=CH—CH_2—COOH,$$

$\alpha]_D^{25} - 130°C$, explodes at 75°C.

This was the second natural ω-acetylene to be discovered, and again is a naturally resolved allene. It is an antibiotic isolated from the fungus *Norcardia acidophilus* by JOHNSON (1947) and its remarkable structure has been proved by CELMER (1952). Mycomycin gives the expected argento compound, and readily isomerizes under mildly alkaline condition with loss of its allene and ω-acetylene structures, all of its optical activity, and most of its antibiotic action, to give isomycomycin.

2. *"Isomycomycin".*

$$CH_3—C\equiv C—C\equiv C—C\equiv C—CH=CH—CH=CH—CH_2—COOH$$

Optically inactive, decomposes slowly at 140°C. This is the alkaline rearrangement product of mycomycin, and has not been found in natural products.

3. *Triene-triynyl acetate from Carlina vulgaris.*

$$CH_2=CH—CH=CH—C\equiv C—C\equiv C—C\equiv C—CH=CH—CH_2OAc.$$

This highly unstable ester was found by SØRENSEN (1954) in the common carline thistle. BOHLMANN (1956) has synthesized the all-trans isomer of M.pt. 31°C, and has indicated that the natural ester is the 10-cis 2-trans isomer.

4. *The Coreopsis poly-ynes.* SØRENSEN (1954) found this garden plant to be an especially rich source of poly-ynes, but the structures given are only tentative:

$$CH_3CH=CH—CH=CH—C\equiv C—C\equiv C—CH=CH—CH=CH_2$$

(in the leaves)

$$CH_3—CH=CH—C\equiv C—C\equiv C—C\equiv C—C\equiv C—CH=CH_2$$

(in the roots)

$$CH_3—C\equiv C—C\equiv C—C\equiv C—C\equiv C—C\equiv C—CH=CH_2$$

(as a trace component)

The pentayne is apparently quite common as a minor component among other Compositae. All parts of the *coreopsis* plants contained in addition a phenyl C_7 diyne,

$$C_6H_5—C\equiv C—C\equiv C—CH=CH—CH_2OAc,$$

and the flowers and roots contained also a phenyl C_{11} triynediene.

The yellow pentayne is exceedingly unstable, except in dilute solution, and is the first natural colouring matter to be found based on a polyacetylenic chromophore.

F. The C_{14} *group*

1. "*Artemisia ketone*".

$$CH_3—C{\equiv}C—C{\equiv}C—C{\equiv}C—CH{=}CH—CH_2—CH_2—COCH_2CH_3$$

was at first thought to be a C_{12} ketone, when isolated impure from *Artemisia vulgaris* (SØRENSEN, 1950) but is now known by both improved isolation and by synthesis (BOHLMANN, 1955) to have the above structure.

2. "*Anacycline*".

$$CH_3CH_2CH_2—C{\equiv}C—C{\equiv}C—CH_2—CH_2—CH{=}CH—CH{=}CH—CONHBu^i$$

(trans-trans, *iso*-Butyl). CROMBIE (1955) found this compound to be a minor component of the sharp melting and reputedly homogeneous pellitorine, obtained from "pellitory root", from the composite *Anacyclus pyrethin*. With LINDLAR'S palladium catalyst this non-toxic diene-diyne gave a highly insecticidal tetrene. With maleic anhydride it readily gave a mono-adduct, extinguishing the diene absorbtion, and allowing the diyne absorbtion to show up.

3. *Anacycline conjugated enediyne*. Crombie found 4 per cent of this as a persistent impurity in even the best samples of anacycline.

G. The C_{17} *group* (*Oenanthe and Cicuta poly-ynes*)

Five closely allied members occur in the roots of the hemlock species *Cicuta virosa* and *Oenanthe crocata* (LYTHGOE, 1953). The two toxins are convulsant poisons, and have caused fatalities among humans and also are a frequent cause of livestock poisoning. Judging from the symptoms, however, the death of Socrates from hemlock was due to the alkaloid coniine in the leaves, and not to the root toxins.

The oenanthe and cicuta groups vary only in the placement of one double bond:

"*Oenanthetol*"

$$n—C_3H_7—CH_2—CH_2—CH_2—(CH{=}CH)_2—(C{\equiv}C)_2—CH{=}CH—CH_2OH$$

"*Oenanotoxine*"

$$C_3H_7—CH_2—CH_2—\underset{\displaystyle OH}{\underset{|}{CH}}—(CH{=}CH)_2—(C{=}C)_2—CH{=}CH—CH_2OH$$

"*Oenanthetone*"

$$C_3H_7—CO—CH_2—CH_2—(CH{=}CH)_2—(C{\equiv}C)_2—CH{=}CH—CH_2OH$$

"Cicutol"

$$C_3H_7—CH_2—CH=CH—(CH=CH)_2—(C\equiv C)_2—CH_2—CH_2—CH_2OH$$

"Cicutoxine"

$$C_3H_7—\underset{\displaystyle OH}{\underset{|}{CH}}—CH=CH—(CH=CH)_2—(C\equiv C)_2—CH_2—CH_2—CH_2OH$$

The structures are probably all-trans, and those of oenanthetol and *dl*-cicutoxine have been proved by synthesis (LYTHGOE, 1955), the *d*-toxine surprisingly being more toxic to mice than the natural laevorotatory material.

VI. ISOLATION AND PHYSICAL PROPERTIES

Natural acetylenes have so far been isolated by purely physical separations. Tariric and isanic acids were discovered because they are the major crystalline acids in their parent oils, Picramnia Sow oil with 95 per cent tariric acid being almost a pure chemical by lipid chemistry standards; all other acetylenic lipids have been recognized largely by the typical ultra-violet absorption shown by the conjugated yne groupings. There is no method at present in use whereby simple unconjugated acetylenes can be readily recognized when present as minor components, though promise is shown by cobalt octacarbonyl (see below).

A. Properties used in separation

The symmetry of the acetylenic group leads to good crystallizing power and high melting point; the group also leads to an increase in boiling point, and as C_{18} mid-chain diynes are surprisingly stable to 200°C, their methyl esters can be concentrated by conventional ester fractionation. The acids follow the saturated acids in lead soap or solvent crystallization procedures. The acetylene group also confers considerable polarity on the molecule, and poly-yne acids tend to concentrate in the more polar phase during countercurrent liquid-liquid distribution, isanic and bolekic acids showing almost the same polarity as ricinoleic acid.

Partition coefficients for system petroleum ether/acetonitrile at — 6°C

(MEADE, 1951)

Solute	$K = \frac{[\text{P.E.}]}{[\text{MeCN}]}$
Me Oleate	14·5
Linoleate	6·8
Linolenate	4·4
Eleostearate	3·1
Stearolate	5·5
Isanate	0·55
Bolekate	0·45
Ricinoleate	0·39
Ricinelaidate	0·43
Ricinstearolate	0·17
Isano OH esters	0·025

The poly-yne group gives a localized stiffness and straightness to the molecule, SØRENSEN's C_{13} enepentayne containing a constrained straight run of twelve carbon atoms, and the cis-eneyne group gives an inflexible bend. Both these elements of molecular shape affect the ability to form urea inclusion compounds; the low stability of the diyne isanic acid complex indicates the important part played by the hydrogen atoms in these complexes, and the failure of the cis-enediyne bolekic acid to form a complex both led to its isolation and was a preliminary proof of its cis-configuration.

B. Infra-red spectra

A single acetylenic linkage gives only weak absorption: as the treble bond is moved from the end to the middle of only a C_7 chain, absorption "weakens and virtually disappears" (WOTIZ, 1949) and little strengthening occurs on conjugation with an olefinic group. This is somewhat remedied by increasing the number of acetylenic linkages, but though the bond is present and drifting from 2210 to 2236 cm^{-1} as unsaturation increases to tetryne, intensities remain low (WHITING, 1952). MEAKINS (1955) reviews a wide range of acetylenes.

The —C≡C— link gives a strong Raman absorption.

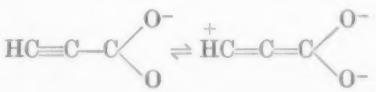
C. Ultra-violet spectra

Again a single acetylene group is almost transparent within the usual bounds of 2200–2800 Å. Conjugation with further acetylenes immediately gives sharp bands of low intensity, and these increase spectacularly in number, sharpness, and intensity with the number of conjugated centres (WHITING, 1952). A nitrile acts much as an extra acetylene. Ene-poly-yne bands are at all times strong enough to mask the purely poly-yne ones, but use of the enormously faster addition of peracetic acid to olefins as against acetylenes allows the masking to be removed, and the lower level poly-yne absorption bands to show up.

SØRENSEN, BRUUN, and HOLME (1954) give the u.v. curve for the tryne dehydromatricaria ester. His 1954 set of papers gives curves of poly-ynes, but mostly for incompletely proven structures. WHITING (1952) gives good penta and hexayne curves, and an interesting graph showing linear dependence of the fundamental u.v. absorption frequency with the degree of unsaturation from diyne to hexayne. BOHLMANN and MANNHARDT (1956) is a further good source of poly-yne u.v. data.

VII. Chemical Properties

The acetylenic group greatly increases the "strength" of a neighbouring carboxyl group, propiolic acid having a dissociation constant 10^3 greater than propionic acid. This effect is limited to acid groups conjugated to acetylene, and has been ascribed to resonance with an allenic structure



This has proved useful in assigning structures to the antibiotic acetylenes. The properties of the carboxyl group are otherwise normal.

Oxygen attacks simple acetylenes far less readily than the corresponding olefins presumably because the α-methylenic attack, which leads to the symmetrical low energy intermediate —CH—CH—CH— in olefins, has no equally powerful driving force in acetylenes. Higher poly-ynes are increasingly unstable to both light and oxygen: the diyne isanic acid has a peculiar instability in the solid state, a red "polymer" forming which does not form with the liquid acid or its liquid esters, though a rapid blue colour formation happens with its crystalline ethanolamide.

Hydrogen, catalysed by nickel, palladium, or platinum, adds to monoacetylenes in two distinct stages, good yields of the cis-olefin being obtained by partial hydrogenation. Conjugated poly-ynes react smoothly, but without definite intermediate stages, no diene being observed in the hydrogenation of isano oil. The lead/quinoline-poisoned palladium catalyst of LINDLAR however, gave a diyne to diene stage in anacycline (q.v.). The use of dibutyl aluminium hydride, which is 97–99 per cent stereospecific in the cis-reduction of hexyne-3 (WILKE, 1956) has not yet been recorded in the acetylenic lipid field.

Halogens react somewhat slowly: bromine adds 50,000 times faster to oleic than to stearolic acid, and further addition to give the saturated tetrabromide is slower still, requiring activation, but normal iodine values for single addition have been recently recorded without indication of any abnormal time requirement. Addition of iodine, which is very incomplete to olefins, is complete with acetylenes, proceeding readily in warm acetic solution in presence of ferrous iron to give stable crystalline di-iodides, which are useful for identification, and have been proposed as X-ray contrast media.

Hydrogen bromide and iodide add doubly to acetylenes giving the halo-olefin and then presumably the gem-dihalo-acid, vicinal di-iodides being very unstable. Thiocyanogen adds reversibly, the maximum thiocyanogen value of stearolic acid obtained at 18°C being 69 instead of the theoretical 91. Hetero-addition of chlorine and methoxy has not been recorded on acetylenic acids, but is known to yield a complex mixture of products on simple acetylenes.

Oxidizing agents act on acetylenes rather less vigorously than on olefins, but only in the simplest cases have details been worked out. Stearolic acid gives good yields of the pale yellow diketoacid with cold dilute permanganate, but hot permanganate or nitric acid give chain cleavage. Peracetic and performic acids act very slowly on simple acetylenes, giving chain cleavage and a little hydration to the ketone: this unreactivity is very marked with eneynes, where addition to the double bonds occurs normally, with very little attack on the acetylenic linkages. This is a valuable weapon in constitutional studies, since it frees the low intensity poly-yne radiation absorptions from the heavy masking action of the strong absorptions due to polyene and eneyne conjugations.

Hydration also has only been applied to simple systems, strong sulphuric acid hydrating stearolic acid to a nearly 1:1 mixture of the 9- and 10-ketostearic

acids. Mercuration leads to hydration, and is a less vigorous reagent, though suspect on grounds of its oxidizing power.

$$—C{\equiv}C— \rightarrow —C(HgOAc) = C(OHgOAc)— \rightarrow —CH_2—CO—.$$

Among miscellaneous reactions, simple acetylenic acids add arsenic trichloride to give chlorvinyl dichlorarsines. Caustic fusion of stearolic acid gives palmitoleic acid and then myristic acid (MANASSE, 1869; confirmed by BODENSTEIN, 1894). Behenolic acid is stable to sodium amalgam and sodium/ethanol, but is reduced by zinc and acid to mixed cis and trans erucic acids. Prolonged treatment with hydrogen iodide leads to complete saturation. Mid-chain acetylenes are stable to lithium aluminium hydride, methyl stearolate giving stearolyl alcohol.

A possible specific reagent for simple acetylenes has at last appeared in the case of cobalt octocarbonyl. GREENFIELD (1956) has found that a wide variety of acetylenes displace the two bridge carbonyls, forming coloured complexes. This should make it possible to examine a wide range of fatty materials for unconjugated acetylenes.

VIII. Physiological Properties

The mid-chain acetylenic linkage in a long chain acid seems to be physiologically innocuous, for stearolic acid has been described as merely "non-nutritive". Anacycline is non-insecticidal until the diyne has been hydrogenated to a diene, and the alkaline rearrangement products of the antibiotics nemotin and mycomycin are of no or low activity.

The terminal ethinyl group is distinctly active. Acetylene itself is regarded as dangerously toxic, the ethnynyl tertiary carbinols are hypnotics, ethinyl steroids show modified but generally increased levels of activity, and the antibiotics nemotin and mycomycin are ω-acetylenes whose activity is largely lost on isomerization.

The convulsant poisons cicutoxine and oenanthotoxine are α-hydroxy diacetylenes, but the similarly constituted isanolic acid from boleko oil is not toxic, so that the toxin activity probably lies in some other unit of the molecular structure. Acetylene conjugated with carboxy is also a physiologically active group, as is shown by the C_8 group antibiotics and by the paralysant activity of propiolic acid, and lesser activity of the substituted propiolic acids.

IX. Synthesis

Many reviews and a recent entire book (RAPHAEL, 1955) cover this excellently. As a résumé, the following methods are generally useful in lipid acetylenic chemistry:

A. $—CH{=}CH— \rightarrow —CHBr—CHBr— \rightarrow —C{\equiv}C—$

This works better with dehydrohalogenation by sodamide in ammonia than by sodium alkoxide, and worse with trans than cis acid dibromides. It is greatly

aided by a β-hydroxy group, as in ricinoleic acid. It fails on linoleic acid tetrabromide:

B. $R—C{\equiv}CH + R'—C{\equiv}CH + Cu_2Cl_2 + O_2 \rightarrow R—C{\equiv}C—C{\equiv}C—R'$.

This is one of the new classical methods and is widely applicable to diyne, higher poly-yne, and enepoly-yne synthesis. The two symmetrical by-products greatly reduce the yield of the crossed oxidation product.

C. $R—C{\equiv}CH + Br(CH_2)_nCl \rightarrow R—C{\equiv}C—(CH_2)_nCl$

This is used as the general synthetic method for all mono-acetylenes, with either the cyanide or the malonate end-play for carboxylic acids. Liquid ammonia is used as solvent. The use of ω-acetylenic esters or of ω-haloesters as intermediates has proved unpromising.

D. $R—CHO + HC{\equiv}C—R \rightarrow R—CHOH—C{\equiv}C—R$

This is another sodamide in liquid ammonia reaction, is applicable to poly-ynes also, and gives alcohols which undergo easy dehydration to conjugated ene-ynes.

E. $R_1—COO^- + R_2—COO^- \rightarrow R_1—R_2 + 2CO_2$

Anodic coupling is the only method of joining up acetylenic fragments away from the acetylenic function. Linstead (1953) developed it and used it for an elegant chain lengthening from stearolic acid to ethyl behenolate. It suffers again from the disadvantage of giving two symmetrical coupling products as well as the desired crossed reaction product.

The above methods give no clue as to the biosynthetic routes, where "high energy" intermediates and mild reaction conditions must apply. Kharasch observed that $\alpha\beta$-difluorsuccinic acid reacted "violently" with water to give acetylene dicarboxylic acid, but that seems to give no help here. Enolpolyphosphates are likelier intermediates.

X. Conclusion

The tally of naturally occurring acetylenes has been rising rapidly in recent years, but only by discovery of conjugated acetylenes. No search whatsoever has been made for simple unconjugated acetylenes, although large differences in chemical and physical behaviour make such a search feasible. The polyacetylenes are very wide-spread in plant material, including fungi, but have not been found in the animal kingdom: their chemical and thermal stability are generally unexpectedly high, though the maximum accumulations of treble bonds leads to explosive instability. Well established methods of synthesis and the high diagnostic value of strong selective energy absorption bands are of great value in this actively developing field.

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3

THE SYNTHESIS OF GLYCERIDES

T. Malkin and T. H. Bevan

CONTENTS

		Page
I.	INTRODUCTION	64
II.	MONOGLYCERIDES	65
III.	1:3-DIGLYCERIDES	69
IV.	1:2-DIGLYCERIDES	72
V.	DIACID DIGLYCERIDES (MIXED DIGLYCERIDES)	73
VI.	TRIGLYCERIDES	74
	A. Monacid or simple triglycerides	74
	B. Diacid triglycerides	74
	C. Triacid triglycerides	74
	REFERENCES	76

3

THE SYNTHESIS OF GLYCERIDES

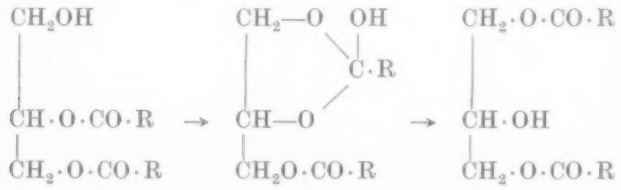
T. Malkin and T. H. Bevan

I. Introduction

Natural fats are mixtures of triglycerides, containing one, two, or three fatty-acid radicals, of which the diacid triglycerides largely predominate. The separation of pure individual glycerides from such complex mixtures is a matter of considerable difficulty, and relatively few have been isolated in the pure state. For the study of the complex physical properties of these compounds (*cf.* Vol. II, p. 1) and also for identification of natural products when isolated, it has been necessary to devise methods of synthesis of the various types. This has engaged the attention of numerous workers, and it is now possible to prepare with relative ease all types of glycerides, with the sole exception of unsaturated 1:2-di-glycerides, which, so far, have defied attempts at synthesis.

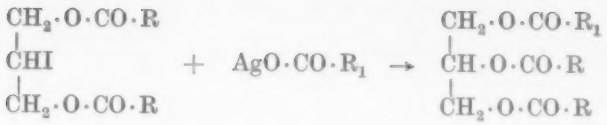
Apart from the monacid triglycerides, which are readily obtained by direct esterification of glycerol, the preparation of other types involves further acylation of mono- and di-glycerides, which are, therefore, key substances in glyceride chemistry. In general, the reactions involved in their preparation are standard methods of protection, acylation, and interchange, but one unusual feature, which has always to be borne in mind in this work, is the possibility of acyl migration. This appears to be of the following two types.

(i) Migration of the 2-acyl group to the 1 position, first observed by Fischer,[1] who suggested the following mechanism:

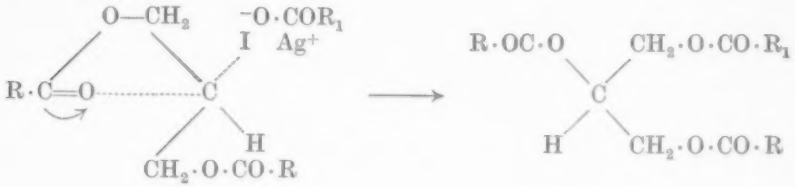


This migration is rapid in the presence of acid, and it is also brought about by alkali or heat. Martin[2] has shown for monoglycerides that an equilibrium is set up favouring 1-monoglyceride, which exists in the mixture to the extent of 90–92 per cent.

(ii) The second type of migration occurs in the opposite direction, i.e. from the 1 to the 2 position, during the interchange between iodides and silver salts of fatty acids.[3] No migration occurs when the above reaction is carried out with 1:2-diacyl glycerol iodide.



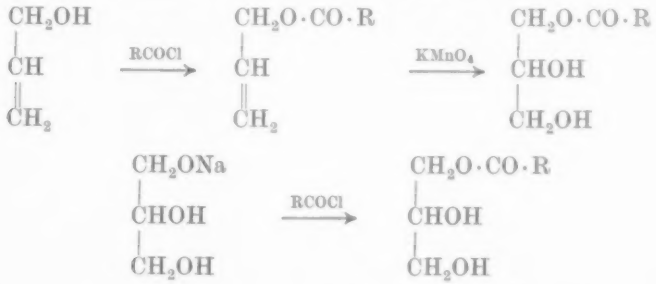
The above migration is similar to that which occurs during the dephosphorylation reaction reported by BEVAN, BROWN, GREGORY, and MALKIN,[4] and probably proceeds as follows:



II. MONOGLYCERIDES

The earliest synthesis of an α-monoglyceride was probably due to BERTHELOT,[5] who heated glycerol with stearic acid for 20 hr at 200°C in a sealed tube. This is roughly the present-day technical method of preparation, which gives rise to a variable mixture of mono-, di-, and tri-glycerides. Other early methods of preparation consisted of heating halohydrins with sodium or potassium salts of fatty acids. These were used by GRÜN and LIMPÄCHER[6] and by ROMBERG,[7] IZAR,[8] GUTH,[9] and KRAFT,[10] but later workers[11, 12, 13] showed that these methods were unsatisfactory because of acyl migration.

A number of other methods, of limited practical value, have been used. These include the following two methods of FAIRBOURNE and associates,[11, 12, 13] starting from allyl alcohol and 1-monosodium glyceroxide.



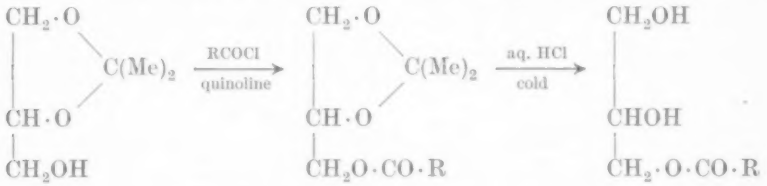
Another rather special method was that of BERGMANN,[14] who proved the constitution of 1-monoglycerides, by preparing 1-monolaurin and 1-monostearin using 2-phenyl-5-chlormethyl oxazolidine as the starting material, and showing that these were identical with those made by FISCHER's method.

Direct esterification methods have been reported by various workers; thus SCHUETTE and HALE[15] prepared 1-monoacetin and 1-monobutyrin by direct esterification of glycerol in the presence of syrupy phosphoric acid, and

similar preparations have been reported by BELLUCCI,[16] GIANOLI,[17] and HILDITCH and RIGG.[18] The difficulty in this method, even when large excess of glycerol is used, is the immiscibility of the fatty acid and glycerol, which results in a slow esterification rate. Once monoglyceride is formed, however, it is more miscible with the acid, and readily esterifies further to di- and tri-glyceride. This method often gives a product which is predominantly diglyceride. It is, of course, possible to separate monoglycerides from the mixture by distillation in vacuo, but on the whole the method is unsuitable for synthetic work.

This also applies to the method of YOUNG and BLACK,[19] who prepared 1-monolaurin by heating trilaurin with glycerol and trisodium phosphate under anhydrous conditions.

By far the best method of preparation, and the one most widely used, is that due to FISCHER, BERGMANN, and BÄRWIND,[20] who used as starting material 1:2-isopropylidine glycerol, which is readily prepared in good yield, from glycerol and acetone, and whose structure was known from the work of IRVINE, MCDONALD, and SOUTAR[21] and later confirmed by HIBBERT and CARTER.[22] This was treated with an acid chloride in the presence of quinoline, and acetone was split off the resulting product by the action of hydrochloric acid in the cold.

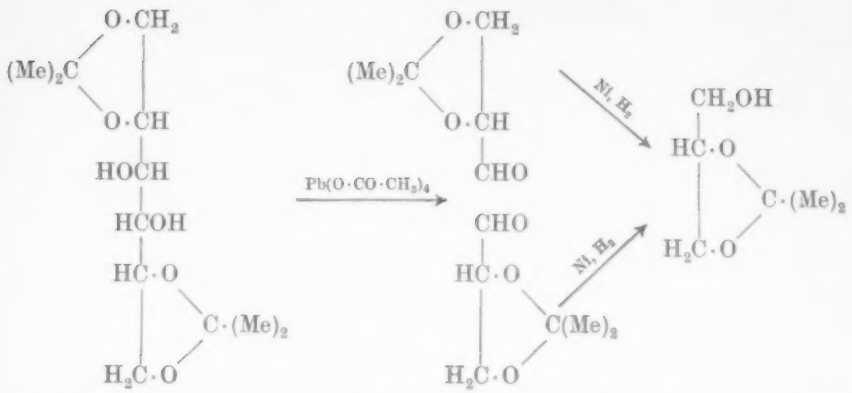


The acylation may be carried out in inert solvents such as benzene or chloroform in the presence of quinoline or pyridine, or the intermediate may be made by passing dry hydrogen chloride into a solution of the fatty acid in isopropylidine glycerol.[23] Excellent yields are obtained by all methods.

High yields are also obtained in the final stage for derivatives of saturated acids higher than C_{12}, but for derivatives of shorter chain acids and unsaturated acids, the resulting monoglycerides are more soluble in the aqueous hydrolysing medium, and there is a tendency to hydrolyse off the fatty acid as well as the acetone. The procedure then becomes a somewhat sensitive compromise, and conditions have to be found so that the amounts of unchanged isopropylidine intermediate and hydrolysed free fatty acids are minimal.

This problem was, however, neatly solved by FISCHER and BAER[24] who found that 10 per cent aqueous acetic acid at 60°C was an admirable reagent for removing the acetone group only. These workers were at the time preparing optically active monoglycerides for which they required the active forms of isopropylidine glycerol. They prepared these according to the following scheme from *D* and *L* 1:2-5:6-di-isopropylidine mannitols.

The intermediate 1-acyl 2:3-isopropylidine compounds may be isolated if required. Those containing the shorter chain acids can be purified by distillation in vacuo, whilst those containing higher acids are purified by crystallization.



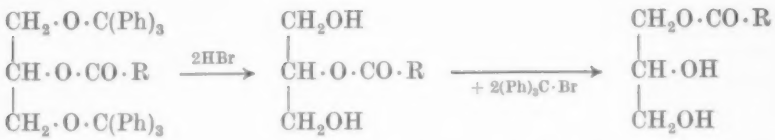
BLACK and OVERLEY[27] applied FISCHER'S method to the synthesis of monolinolein by treating isopropylidine glycerol with tetrabromostearoyl chloride and debrominating with zinc after the final acid hydrolysis.

A similar method to FISCHER'S has been used by DAUBERT and KING,[28] who used 1:2-benzylidine glycerol as the starting material instead of isopropylidine glycerol. This was acylated with the appropriate acid chlorides in pyridine, and the resulting intermediates, which were easily purified by crystallization, were hydrolysed by cold aqueous hydrochloric acid, to give very high yields of 1-monoglycerides.

2-Acyl-1:3-benzylidine glycerol had earlier been used for the preparation of 2-monoglycerides by BERGMANN and CARTER[29] and STIMMEL and KING,[30] as hydrogenation in the presence of a palladium catalyst removed the benzylidine group as toluene. In this case, acid hydrolysis is precluded because of acyl migration, which would result in almost complete transformation into the 1-isomer.

In view of the fact that catalytic reduction of 2-acyl-1:3-benzylidine glycerols give 2-monoglycerides in good yield, there is no reason to doubt that catalytic reduction of 1-acyl-2:3-benzylidine glycerol would equally give a good yield of 1-monoglyceride, although, of course, this method could not be used for the preparation of unsaturated monoglycerides. The benzylidine method, therefore, offers no obvious advantage over that of FISCHER, for the preparation of 1-monoglycerides, but it is a valuable method for the preparation of 2-monoglycerides. An important paper on the preparation of isomeric benzylidine glycerols is due to VERKADE and VAN ROON.[31] The trityl (triphenylmethyl) group has been widely used in glyceride synthesis, particularly by VERKADE, who has made this field his own and who has published an excellent summary of his work.[32] Both 1-monotrityl and 1:3-ditrityl glycerol are prepared in good yield, by the action of trityl chloride on glycerol, in the presence of pyridine,[33] and these are readily acylated in high yield to give highly crystalline compounds. Detritylation can then be brought about by treatment with a saturated solution of hydrogen bromide in glacial acetic acid[34] or, still better, by gaseous hydrogen chloride in ether.[35] Acyl-1:3-ditrityl compounds will, therefore, give rise to

2-monoglycerides, which are at once transformed by acyl migration into 1-monoglycerides.

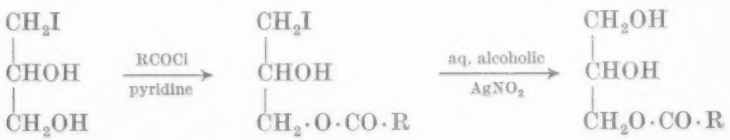


HELFERICH and SIEBER,[34] who were the first to use the above reaction, did in fact obtain 2-monoglycerides of certain aromatic acids, since these do not undergo migration so readily as the aliphatic compounds.

In order to avoid migration, DAUBERT,[36] following a suggestion of VERKADE, VAN DER LEE, DE QUANT, and DE ROY VAN ZUYDEWIJN[37] removed the trityl groups by catalytic hydrogenation in the presence of palladium black or platinum oxide, although VERKADE[32] prefers palladium on carbon to platinum oxide, because of the traces of alkali invariably retained by the latter. VERKADE and VAN DER LEE[38, 39, 40] have shown that 1-monotrityl glycerol is readily acylated in the 3 position to give easily purified crystalline products, and it would appear that these could be readily detritylated, either by halogen acids or catalytically, to give good yields of monoglycerides. Naturally, detritylation by catalytic reduction cannot be employed if unsaturated glycerides are required.

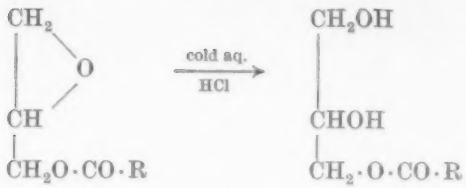
Although the stages of the trityl method of synthesis proceed in high yield, it cannot compete with the methods of FISCHER, BERGMANN, and BARWIND, and BERGMANN and CARTER, for the preparation of 1- and 2-monoglycerides. This is because of the production of triphenylhalide or triphenylmethane in the detritylation stage, which, particularly for the shorter chains, and hence more soluble glycerides, is removed only by a somewhat wasteful crystallization.

A method which we have used successfully in our laboratories, and which was first reported by GRÜN and WOHL,[41] employs glycerol 1-iodohydrin as starting material. This is acylated in good yield in the presence of pyridine or quinoline, to give an intermediate, which is easily purified by crystallization. On warming this intermediate with aqueous alcoholic silver nitrite, the iodine atom is replaced by hydroxyl. We have obtained yields of monopalmitin of the order of 80 per cent. This method is an extension of FISCHER'S well-known method for the preparation of 1:3-diglycerides.[42]



KESTER, GAISER, and LAZAR[43] have reported the preparation of 1-monolaurin by the hydrolysis of the oxide ring in lauryl glycidol, by aqueous hydrochloric acid, but no details or yields are given.

1:3-Diglycerides

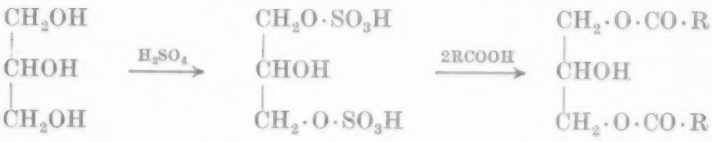


An outstanding problem in glyceride synthesis which has long defied solution, namely the preparation of unsaturated 2-monoglycerides, has now been elegantly solved by MARTIN.[44] It will be recalled that 2-monoglycerides are made from 2-acyl-1:3-benzylidine glycerols by hydrogenolysis, a process which would, of course, reduce any unsaturated acyl group. On the other hand, if the benzylidine group is removed in the conventional manner with hydrochloric acid, the final product is the 1-monoglyceride. MARTIN found that if the 2-acyl-1:3-benzylidine compound is heated for a short time with boric acid in ethyl borate, the benzylidine group is removed and the 1:3-hydroxyl groups are esterified to borates. The borates groups are immediately hydrolysed on contact with water to yield a symmetrical monoglyceride.

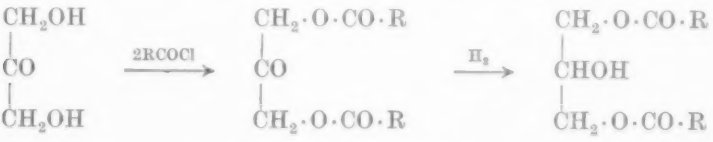
III. 1:3-DIGLYCERIDES

As in the case of monoglycerides, the first synthesis was probably due to BERTHELOT,[5] who heated stearic acid with glycerol in a sealed tube at 200° and who probably obtained a mixture of mono-, di-, and tri-stearin.

GRÜN and SCHACHT[45] reported the preparation of 1:3-dimyristin by heating symmetrical glycerol disulphate with myristic acid dissolved in sulphuric acid.



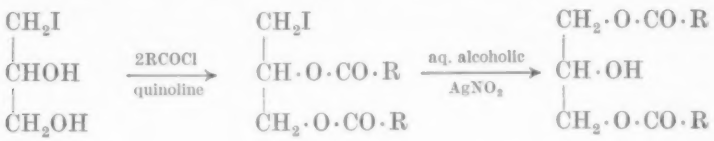
and GRÜN and WITTKA[46] reported a synthesis from dihydroxy acetone



A number of other workers, including GUTH,[9] have prepared diglycerides by the action of sodium and potassium salts of fatty acids on dibromo- or dichlorohydrins, but as was mentioned earlier, such reactions give anomalous results owing to the possibility of acyl migration.

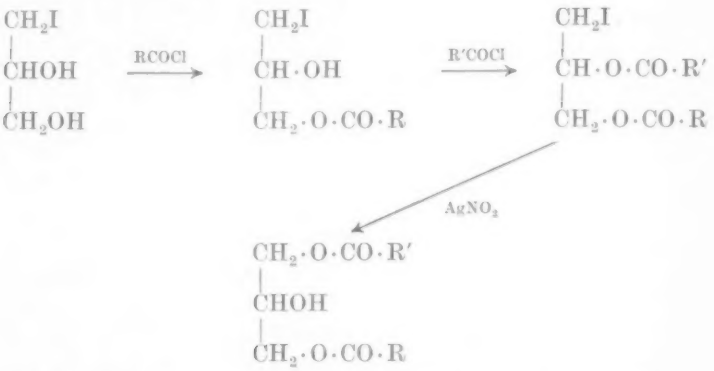
All these earlier methods are unsatisfactory in one respect or another, and the first reliable method for the preparation of 1:3-diglycerides is due to FISCHER,[42] who acylated glycerol 1-iodohydrin with 2 moles of acid chloride in the presence

of quinoline (pyridine serves equally well) and refluxed the readily purified, crystalline product with aqueous alcoholic silver nitrite.

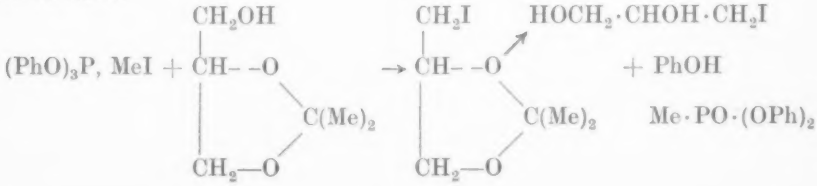


In the final stage, hydrolysis of the iodide and acyl migration occur.

The method is readily adapted to the preparation of diacid 1:3-diglycerides (GRÜN and WOHL[41] and KIRCH[47]) for acylation with a mole of acid chloride, yields the 3-acyl 1-iodo-compound, which can be purified by crystallization and further acylated with a different acid chloride. Treatment with silver nitrite then yields a mixed 1:3-diglyceride.



This method gives excellent yields of pure products, and it is, in our view, still the best, for the production of 1:3-diglycerides. Until quite recently, the only objection to its general use was the unavailability of the iodohydrin. FISCHER and PFÄHLER[48] prepared this in 29 per cent yield by heating 1:2-isopropylidine-3-chlorohydrin, dissolved in alcohol, with sodium iodide in a sealed tube at 100°, for three days, followed by hydrolysis of the acetone residue. More recently, FISCHER and BAER[49] considerably increased the yield by replacing the toluene-*p*-sulphonyl group in 2:3-isopropylidine dioxypropyl toluene *p*-sulphonate, by heating with sodium iodide in acetone in a sealed tube, and, still more recently, BEVAN, MALKIN, and SMITH[50] obtained high yields by the following reaction, due to LANDAUER and RYDON,[51] which obviates the use of sealed tubes.



Slightly earlier, VERKADE, HESSEL and VAN LOHUISEN[52] also showed that the iodohydrin could be prepared in high yield, by the action of constant boiling hydriodic acid on glycidol at 10°C.

$$CH_2OH \cdot CH \cdot CH_2 + HI \rightarrow CH_2OH \cdot CHOH \cdot CH_2I$$
$$\qquad\qquad\quad \diagdown\diagup \quad O$$

Because of the unavailability of the iodohydrin, MALKIN, SHURBAGY, and MEARA[53] explored other methods and obtained good yields of 1:3-diglycerides by acylation and by direct esterification of 1-monoglycerides. The important practical point in both methods is to use some 5–10 per cent excess of monoglyceride in order to avoid formation of triglyceride.

Excess of monoglyceride in the final product is readily removed from the much less soluble diglyceride by crystallization from alcohol, but triglyceride can be removed completely only with great difficulty.

The acylation is best carried out by the addition of a slight excess of pyridine to a dry benzene solution of the monoglyceride and acid chloride, preferably with mechanical agitation. The direct esterification is carried out by heating the monoglyceride and acid at 120–140°C for a few hours, in the presence of *p*-toluene- or *d*-camphor- 10-sulphonic acid, water being removed from time to time by brief attachment to the vacuum pump. Both methods give yields of 65–85 per cent of purified diglyceride, but the second method is best confined to the less volatile acids, C_{14} upwards.

An excellent method of preparation, which has been widely used by many workers in the glyceride field, and which, particularly for diglycerides of the higher fatty acids, i.e. C_{14} upwards, competes favourably with the method of FISCHER in its general convenience, and quality and yield of product, is that developed by VERKADE, VAN DER LEE, and MEERBURG.[35] These workers acylated the 1-monotrityl ether of glycerol, which is easily prepared, and removed the trityl groups by treatment with a solution of dry hydrogen-chloride gas in ether or petrol ether. The authors prefer the latter detritylation reagent to hydrogen bromide in acetic acid, since it increases the yield, and avoids the formation of small amounts of brominated by-products. The only objection to this synthesis is that, for the more soluble diglycerides, the removal of the triphenylhalide by-products by crystallization brings about a loss in yield.

$$\begin{array}{l} CH_2 \cdot O \cdot C(Ph)_3 \\ | \\ CHOH \\ | \\ CH_2OH \end{array} \xrightarrow[\text{pyridine or quinoline}]{2RCOCl} \begin{array}{l} CH_2 \cdot O \cdot C(Ph)_3 \\ | \\ CH \cdot O \cdot CO \cdot R \\ | \\ CH_2 \cdot O \cdot CO \cdot R \end{array} \xrightarrow[\text{ether}]{HCl\ gas} \begin{array}{l} CH_2 \cdot O \cdot CO \cdot R \\ | \\ CH \cdot OH \\ | \\ CH_2 \cdot O \cdot CO \cdot R \end{array}$$

This method has been used by DAUBERT and LUTTON[54] for the preparation of 1:3-diglycerides of oleic, linoleic, and linolenic acids.

ROSE[55] claims a yield of 67 per cent of pure 1:3-dipalmitin by the direct acylation of glycerol with 2 moles of acid chloride in chloroform containing pyridine or quinoline. Our experience with this reaction is that the product

invariably contains triglyceride, which is difficult to remove. A reasonably pure product may occasionally be obtained, but on the whole the method is too unreliable for general use, and it does not, of course, lend itself to the preparation of diacid diglycerides.

A method, starting from glycidol, has been used by HUNTER, ROBERTS, and KESTER,[56] who heated 1-glycidyl stearate with erucic acid in chlorobenzene for 28 hr. The yield of mixed glyceride was about 40 per cent and the method therefore offers no advantage over others.

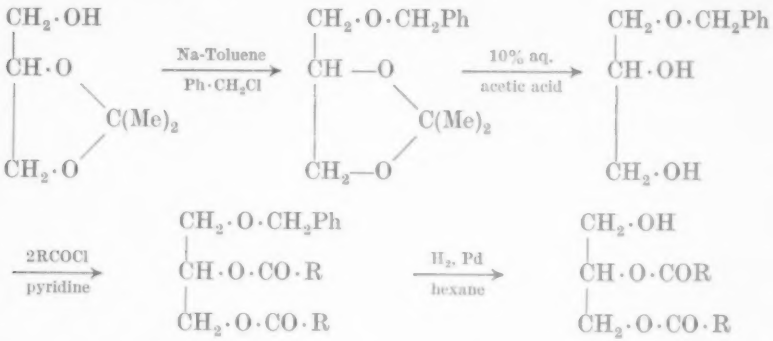
BAUR and LANGE[57] have described a preparation of 1:3-diglycerides, which is a modification of the interesterification-alcoholysis process of ECKEY[58] and ECKEY and FERMO,[59] previously used for the preparation of both mono- and di-glycerides. A mixture of a pure single-acid triglyceride and triacetin is heated with a 10 per cent excess of the theoretical amount of dry glycerol to convert all the triglycerides present into diglycerides, and 0·5 per cent sodium methylate. The whole is maintained above the complete melting point of the mixture until interesterification and randomization is complete. On cooling slowly the 1:3-diglyceride separates out in a high state of purity. The use of triacetin, which is a new feature in the above reaction, permits a lower reaction temperature, and appears to facilitate the crystallization of the product. Starting with a pure triglyceride, the method gives high yields of pure products, although the claim that the purity is greater than that given by other methods is open to question.

Recently published syntheses by BARRY and CRAIG[79] from dihydroxyacetone and allyl alcohol, and by PORCK and CRAIG,[60] from 2-O-benzylglycerol, are of academic interest.

IV. 1:2-DIGLYCERIDES

The preparation of these compounds is a matter of some difficulty because of the ready migration of the 2-acyl group, and consequently the usual acid reagents for removing protecting groups cannot be used. The only satisfactory methods of preparation involve (1) the protection of one primary hydroxyl group of glycerol; (2) complete acylation; (3) removal of the protecting group by catalytic hydrogenolysis. VERKADE, VAN DER LEE, DE QUANT, and DE ROY VAN ZUYDEWIJN[37] and VERKADE, COHEN, and VROEGE[60] effected protection with the trityl group, which they removed with palladium on activated charcoal. This method is satisfactory for higher members, but for lower members, removal of the by-product triphenylmethane incurs crystallization losses. DAUBERT

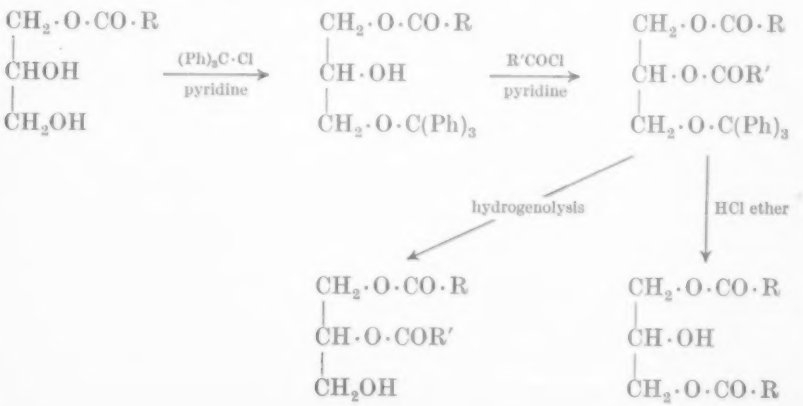
and KING[61] avoided this by protection with the carbobenzoxy group, which on hydrolysis is converted into toluene, but unfortunately the intermediate carbobenzoxy glycerol decomposes on distillation, and is difficult to obtain pure. Later, SOWDEN and FISCHER[62] used the 1-benzyl ether of glycerol in the preparation of optically active 1:2-diglycerides, and HOWE and MALKIN[63] were able to improve this method according to the following scheme, which gives a high yield at each stage.



All the above methods are readily adapted to the preparation of diacid 1:2-diglycerides, but not of course to the preparation of unsaturated 1:2-diglycerides. So far, the latter have never been synthesized, and this remains the major outstanding problem in glyceride synthesis.

V. Diacid Diglycerides (Mixed Diglycerides)

All the methods starting from a 1-protected glycerol (including monoglycerides and glycerol 1-iodohydrin) can be used for the preparation of mixed diglycerides by acylating in stages with different acyl chlorides or acid. 1-Protected glycerol acylates preferentially in the 3 position, and conditions should be such as to avoid acylation in the 2 position as well.



For derivatives of the higher fatty acids, the method of VERKADE and VAN DER LEE[39] is probably the best. These workers tritylate a 1-monoglyceride, since trityl chloride appears to have a greater preference for attacking the primary hydroxyl than most acyl chlorides. The purified product is then fully acylated and detritylated either under acid[39] or neutral conditions (VERKADE, COHEN, and VROEGE[60]).

This method has been used by CHEN and DAUBERT,[64] SIDHU and DAUBERT,[65] and DAUBERT and LONGENECKER.[66]

An equally good method for 1:3-diglycerides is the method of GRÜN and WOHL[41] and GRÜN and KIRCH,[47] who used the iodohydrin method and acylated in stages with different acyl groups. As indicated earlier, the first stage of this reaction proceeds satisfactorily in good yield (see p. 68).

VI. TRIGLYCERIDES

(*a*) *Monacid or simple triglycerides*

These are readily prepared by heating a small excess of the theoretical amount of fatty acid with glycerol, at 140–150°C for a few hours in the presence of 1–2 per cent of *p*-toluenesulphonic acid. Alternatively, and preferably, if the acids are unsaturated, glycerol is acylated in the presence of excess of pyridine or quinoline, with three moles of acid chloride. Representative preparations are found in the work of CLARKSON and MALKIN,[67] VERKADE, VAN DER LEE, and Mlle. MEERBURG,[68] and DAUBERT and BALDWIN.[69]

(*b*) *Diacid triglycerides*

The *unsymmetrical* diacid triglycerides are prepared by acylating 1-monoglycerides with two moles of acid chloride in the standard manner. The method has been extensively used by MCELROY and KING,[70] CARTER and MALKIN,[71] and DAUBERT and BALDWIN.[72]

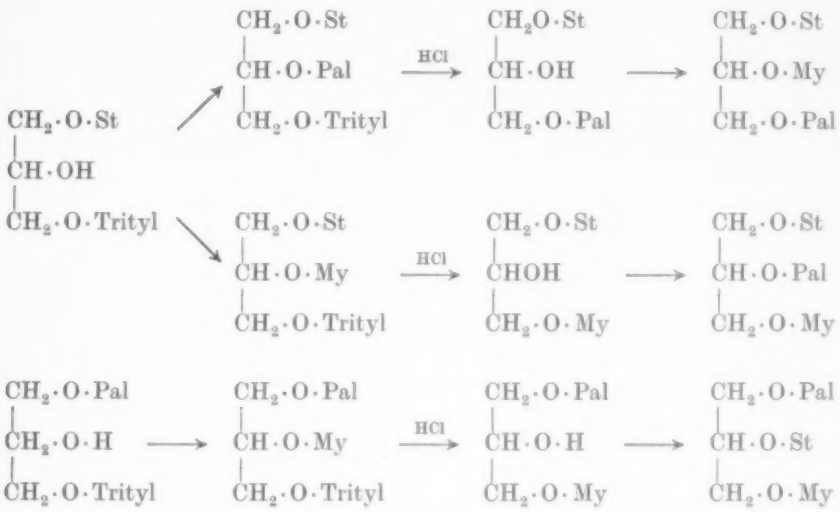
It is, of course, possible to prepare these compounds by the acylation of 1:2-diglycerides, or as mentioned in the introduction, by the interaction of a silver salt of a fatty acid and 1:3-diacyl-2-glycerol iodohydrin. These methods, however, obviously cannot compete in simplicity with the former method.

The *symmetrical* diacid triglycerides are made by the acylation of 1:3-diglycerides in the standard manner. It is advisable to use a moderate excess of acid chloride, and when using unsaturated acid chlorides, a large excess and an elevated temperature may be required. Excess of acid in the final product is easily removed by crystallization from alcohol in which triglycerides are only slightly soluble. The above method has been used by FISCHER,[1] AVERILL, ROCHE, and KING,[73] MALKIN and MEARA,[74] JACKSON, DAUBERT, KING, and LONGENECKER,[75] and MALKIN and WILSON.[76]

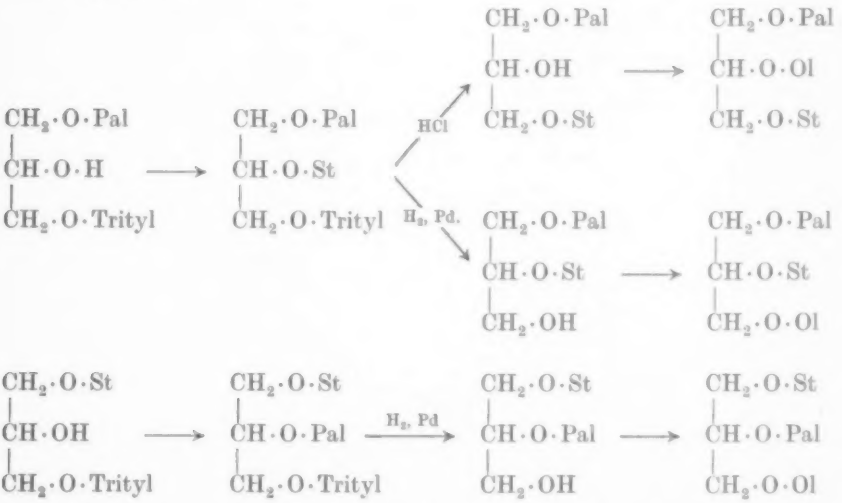
(*c*) *Triacid triglycerides*

These have been prepared by the acylation of diacid diglycerides in the standard manner by VERKADE, VAN DER LEE, and Mlle. MEERBURG,[77] VERKADE,[78] GRÜN and WOHL,[41] CHEN and DAUBERT,[64] and SIDHU and DAUBERT.[65]

VERKADE *et al.* obtained 70 per cent yields based on the starting material of the isomeric glycerides containing the myristoyl, palmitoyl, and stearoyl radicals, according to the following scheme.



VERKADE[78] also prepared oleoyl, palmitoyl, stearoyl glycerols according to the following scheme.



GRÜN *et al.*[41, 47] has prepared triacid triglycerides by prolonged heating at 100–120°C a mixture of the sodium salt of a fatty acid with a diacid diacyl glycerol iodohydrin. This particular interchange probably proceeds without migration (see introduction), and it would be improved by using the silver salt.

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4

LIPID WAXES

A. H. Warth

CONTENTS

		Page
I.	INTRODUCTION	80
II.	THE ALCOHOLS OF LIPID WAXES	80
	A. Physical properties of *n*-alcohols	81
	1. Melting and boiling points	81
	2. Densities	81
	3. X-ray spacings	81
	B. Occurrence of wax alcohols in nature	82
	C. Separation of *n*-alcohols	82
	1. Fractional distillation of alcohols	82
	2. Fractionation distillation of acetylated alcohols	82
	3. Amplified distillation technique	82
	D. Iso and ante-iso alcohols	83
	1. Degradation of the alcohols	83
	E. Dihydric alcohols	84
	F. Sterols and triterpene alcohols	85
	1. Determination of triterpene alcohols	85
	2. Amyrins and lupeol	86
III.	THE ACIDS OF LIPID WAXES	87
	A. Physical properties of fatty acids	87
	B. Separation of *n*-acids by methylation	87
	C. Iso and ante-iso acids	88
	D. Hydroxy acids	89
	1. Saturated monohydroxy acids	89
	2. Dihydroxy acids	90
	E. Lactones and lactides	90
	1. δ-lactones in wool wax	90
	F. Aromatic acids	92
IV.	THE ESTERS OF LIPID WAXES	93
V.	THE HYDROCARBONS OF LIPID WAXES	94
	REFERENCES	95

4

LIPID WAXES

A. H. Warth

I. Introduction

Lipid waxes are widely distributed in nature in both the vegetable and animal kingdoms. The lipid waxes are essentially esters of aliphatic alcohols combined with carboxylic acids. They usually contain a small amount of free fatty acid, and hydrocarbons. A few hard vegetable tallows, notably bayberry wax, are included in the category of lipid waxes. Some of the lipid waxes also contain polycyclic alcohols, such as sterols and triterpene alcohols, in the combined and free states.

The aliphatic alcohols are usually normal saturated monohydric alcohols having an even number of carbons ranging from C_{10} to C_{44}, although seldom are alcohols encountered which have more than 32 carbons. Jojoba wax, expressed from the seeds of *Simmondsia chinensis* (*californica*), is one of the few known liquid waxes. It has, as a lipid component, esters of unsaturated alcohols (C_{20}—C_{26}) combined with unsaturated fatty acids (C_{16}—C_{22}).

II. The Alcohols of Lipid Waxes

When a wax is saponified by adequate treatment with caustic alkali in a suitable solvent, followed by the addition of an excess of water, the unsaponifiable matter may be separated by a selective solvent. In general, the unsaponifiable matter consists of hydrocarbons and wax alcohols, the latter predominating except in candelilla wax.

Analytically, the wax alcohols are destroyed by treatment with fuming hydrochloric acid, leaving the hydrocarbons intact. Many of the lipid waxes yield 50 to 55 per cent of wax alcohols, free and combined (as esters), whereas the fats yield only 1 or 2 per cent of fat alcohols, since the glycerol (trihydric alcohol) produced by the hydrolysis of fats is water-soluble. The normal fat alcohols in nature all have an even number of carbon atoms.

A. Physical properties of n-alcohols

1. *Melting and boiling points.* In Table 1 are presented data relating to the melting and boiling points at various pressures of solid normal saturated monohydric aliphatic alcohols of synthetic origin.

References to the original investigations are given in the footnotes.

Table 1. Solid normal saturated monohydric aliphatic alcohols of synthetic origin ($C_nH_{2n+2}O$)

C number	*Systematic name*	*Common name*	*M.pt.* (°C)	*B.pt.* (°C) at 750 *mm*	at 15 *mm*	at 0·25 *mm*
10	*n*-decanol	capryl	6·0[a]	232[g]	120_{12}[h]	—
12	*n*-dodecanol	lauryl	23·9[a]	257[g]	150_{20}[h]	—
14	*n*-tetradecanol	myristyl	37·6[a]	286[g]	$171·5_{20}$[h]	—
16	*n*-hexadecanol	cetyl	49·1[b]	—	190[h]	—
18	*n*-octadecanol	stearyl	57·9[a]	—	210[f]	153·5
				15 *mm*	0·25 *mm*	0·02 *mm*
20	*n*-eicosanol	arachic	65·3[c]	220[h]	$178·_{40}$[h]	—
22	*n*-docosanol	behenyl	70·6[d]	—	$180·_{23}$[h]	—
24	*n*-tetracosanol	lignoceryl	75·3[e]	—	$210·_{40}$[h]	—
26	*n*-hexacosanol	ceryl	79·2[e]	—	—	—
28	*n*-octacosanol	montanyl	82·6[e]	—	—	175
					0·5 *mm*	
30	*n*-triacontanol	myricyl	86·8[f]		244	
32	*n*-dotriacontanol	lacceryl	89·2[e]		257	
34	*n*-tetratriacontanol	geddyl	91·7[e]		267	

[a] Meyer and Reid (1953) (α form stable, α-crystal freezing point corresponds to lowest melting point). [b] Delcourt (1931); [c] Adam and Dyer (1925); [d] average of 71, 70·8, 70–70·4, and 70·5, of four investigators; [e] Francis, Collins, and Piper (1937); [f] average of 87·5, 86·6, and 86·5, of three investigators; [g] Schon (1952) (mean of range reported); [h] as adapted by Ralston (1948); [i] Levene *et al.* (1915).

2. *Densities of n-alcohols.* The densities (d_4^t) of the *n*-alcohols which occur in lipid waxes are given in Table 2 for their melted state. Temperature in °C is given in parenthesis.

Table 2. Densities of n-alcohols

C_{10}	0·8297 (20)[a]	C_{22}	0·8000 (73)[c]
C_{12}	0·8309 (24)[a]	C_{24}	0·7950 (77)[c]
C_{14}	0·8236 (38)[a]	C_{26}	0·7890 (82)[c]
C_{16}	0·8197 (50)[b]	C_{28}	0·7830 (85)[c]
C_{18}	0·8124 (59)[a]	C_{30}	0·7770 (88)[c]
C_{20}	0·8060 (67)[c]	C_{32}	0·7710 (91)[d]

[a] As listed by Ralston (1948); [b] Delcourt (1931); [c] as listed by Warth (1947); [d] computed value.

3. *X-ray spacings.* The recorded crystal spacings (in Ångstrom units) for melted layers of *n*-alcohols are essentially those of B spacings obtained by Francis, Collins, and Piper (1937), in which the alcohols above C_{28} were purified by molecular distillation, and the lower ones by distillation at 10^{-5} mm pressure. The determination of crystal spacings is set forth by Malkin in Volume I of this series.

B. Occurrence of wax alcohols in nature

Cetyl alcohol ($C_{16}H_{34}O$) occurs in the combined state as cetyl palmitate, and to a lesser extent as cetyl myristate and cetyl laurate in spermaceti wax. Cetyl alcohol (lethal) was discovered by CHEVREUL over a century ago. It can now be prepared cheaply from ethyl palmitate by hydrogenation, and is of considerable use in the cosmetic industry. It crystallizes from alcohol in leaflets which melt at 49–50°C. At the other end of the series of wax alcohols is the C_{32} alcohol, dotriacontanol ($C_{32}H_{66}O$), recovered from carnauba wax by MURRAY and SCHOENFELD (1951). It was isolated by saponification of the fractionated acetylated nonsaponifiable matter after chromatographing on alumina and crystallizing from petroleum ether. It consists of large white laminae having a melting point of 89·2 to 89·6°C, and a solidification point of 88·8°C.

C. Separation of n-alcohols

1. *Fractional distillation of alcohols.* KOONCE and BROWN (1944) stated that it was probable that carnauba wax (hydrolysed) contains straight-chain alcohols of even carbon number from C_{26} to C_{36}—the higher alcohols dominating —and a dihydric alcohol, possibly $C_{23}H_{46}(CH_2OH)_2$. The monohydric alcohols, however, had never been isolated in a pure condition. This these investigators attempted to do. KOONCE and BROWN (1944) fractionally distilled the alcohols, and from the melting points of the alcohols and their derivatives concluded that the alcohols, C_{28}, C_{30}, and C_{32} were the chief alcohol components. Lacceryl alcohol, C_{32}, which had been isolated like the others in a purity of 95 per cent, was regarded as the major constituent. Seven years later MURRAY and SCHOENFELD (1951) investigated the composition of the nonsaponifiable portion (wax alcohols) of carnauba wax using a modified spinning-band column developed in the laboratory and described by MURRAY (1951). This column had a very low pressure drop and high efficiency at low operating pressures suitable for the fractionation of such high boiling material.

2. *Fractional distillation of acetylated alcohols.* The cleaned wax (200 gm) was saponified, the wax alcohols were taken up with petroleum ether and after purification the recovered alcohols were of pale yellow colour, melted from 84·8 to 85·5°C, and amounted to 53·5 per cent of the wax. The acids recovered represented 96·8 per cent of the calculated yield. A brown resinous material, probably resin acids, amounted to 4·6 per cent. The alcohols were acetylated and fractionated in a spinning band column with a rotor length of 366 cm at a pressure of 0·50 mm. Twenty-two fractions were collected. Combined fractions were saponified and the recovered alcohol chromatographed on alumina at 50°C then crystallized from petroleum ether. The crystals were obtained as large laminae melting point 89·2 to 89·4, solidification point 88·8°C. There was close agreement between the values and those of synthetic dotriacontanol (C_{32}) published by PIPER *et al.* (1934).

3. *Amplified distillation technique.* Since the lower fractions could not be

isolated with assurance of purity, KOONCE and BROWN resorted to an amplified distillation technique in the separation of the acetates. In this technique the acetylated alcohols are fractionally distilled with an amplifying agent which consists of a mixture of mineral oil and ceresin wax. Acetylated alcohols distil with hydrocarbon oils without the formation of azotropes. The technique is similar to that employed by WEITKAMP (1947) in the separation of fatty acids.

D. Iso and ante-iso alcohols

Many of the alcohols isolated from natural waxes have lower melting points than synthetic normal alcohols of the corresponding molecular weight; and this also applies to their derivatives. Some of the more recent investigations indicate that *n*-alcohols are often intimately combined, perhaps dimerically, with iso alcohols, and the separation of such alcohols when of high molecular weight is always difficult.

The chemical literature in the past has referred to the aliphatic alcohols of the unsaponifiable substance of wool wax as *n*-alcohols and lano alcohols. The latter could not be identified further than the number of carbon atoms, a terminal OH group, and a melting point differing from that of the corresponding *n*-alcohol. For example, the lano-octadecyl alcohol of KUWATA and KATUNO (1938) had a melting point of 42–43°C; in all probability it was a mixture of iso and ante-iso C_{18} alcohols. Ante-iso alcohols have the methyl side linkage in the preultimate position of the chain.

It was not until WEITKAMP (1945) elucidated the structure of the acids in the esters of wool wax as normal, iso and ante-iso, that it was realized the alcohols of wool wax might be similar. It remained for MURRAY and SCHOENFELD (1951) to elucidate the composition of the isomers of the alcohols. TIEDT and TRUTER (1952) isolated straight-chain alcohols corresponding to the *n*-acids described by WEITKAMP. They separated by means of their urea adducts five normal alcohols, namely C_{18}, C_{20}, C_{22}, C_{24}, and C_{26}. These alcohols constitute about 7 per cent of the composition of wool wax.

1. *Degradation of the alcohols.* The starting material used in the study by MURRAY and SCHOENFELD (1951) was extracted with trichloroethylene from a bale of authentic merino sheep wool. They removed the triterpene alcohols from the unsaponifiable matter by virtue of their low solubility in methanol. Cholesterol was separated as its addition compound with anhydrous zinc chloride. After the triterpene alcohols were filtered off and the cholesterol separated, the residual alcohols (80·6 per cent) were reacted with acetic anhydride, purified, and fractionally distilled. For the distillation they chose a column of the spinning band type of MURRAY, which has a lower pressure drop than the Stedman type used by WEITKAMP and permits normal alkyl acetates up to C_{34} to be distilled. The alcohols were then obtained by saponification of the acetates. In this manner these investigators were able to isolate six dextrorotatory ante-iso alcohols with odd-numbered chains ranging from C_{17} to C_{27} and four optically inactive iso alcohols with even-numbered chains ranging

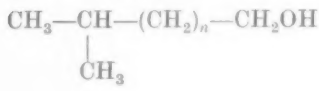
from C_{20} to C_{26} from wool wax. Melting points are given in Table 3. The ante-iso alcohols show optical rotations ($[\alpha]_D^{23}$, in $CHCl_3$) of 4·4–3·5°C.

Table 3. Melting points of branched alcohols

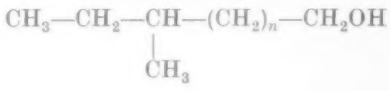
Ante-iso series	*M.pt. (°C)*	*Iso series*	*M.pt. (°C)*
14-methyl hexadecanol	18·4	18-methyl nonadecanol	50·3
16-methyl octadecanol	27·5–31	20-methyl heneicosanol	57·1
18-methyl eicosanol	40·1	22-methyl tricosanol	62·4
20-methyl docosanol	48·5	24-methyl pentacosanol	67·2
22-methyl tetracosanol	54·6		
24-methyl hexacosanol	61·0		

Eighty-five per cent of the acyclic alcohols of wool wax are within the range of C_{20}—C_{27}. The structural formulas for the iso alcohols and ante-iso alcohols are respectively as follows:

Iso series



Ante-iso series



E. Dihydric alcohols

A few of the lipid waxes contain acyclic dihydric alcohols. Horn and Hougen (1951) isolated five alkyl-1, 2-diols from wool wax. They have an even number of carbon atoms and are in the range of C_{18} to C_{24} inclusive, and their properties indicate that their alkyl chains have a single methyl branch.

Murray and Schoenfeld (1951) observed that the lower fractions obtained in the simple fractionation of the acetates of the alcohols of carnauba wax have a different crystalline appearance and high saponification values, indicating the presence of glycols in these fractions. The acetates were accordingly saponified and the recovered alcohol was absorbed on activated alumina at 50°C and eluted in turn with benzene and acetone. The material eluted by acetone was crystallized from benzene and obtained as small dendrite crystals. Small amounts of two higher melting alcohols were obtained from other fractions. Analysis of C, H, and O of these alcohols indicated a homologous series of glycols differing by two carbons. The series includes:

	M.pt. (°C)	*Solidification point (°C)*
$C_{21}H_{42}(OH)_2$ . . .	105·5–105·8	105·4
$C_{23}H_{46}(OH)_2$ . . .	108·4–108·6	108
$C_{25}H_{50}(OH)_2$ . . .	109·6–110·2	109·6

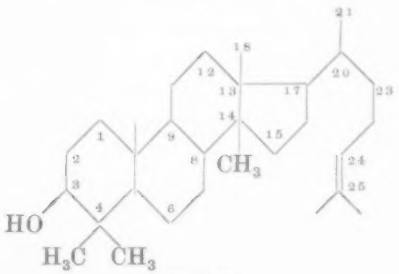
The glycols in the unhydrolysed carnauba wax are combined as acid esters and diesters, as was shown later by the functional group computations by FINDLEY and BROWN (1953).

F. Sterols and triterpene alcohols

A few of the plant waxes contain mixed esters of sterols and triterpene alcohols in addition to esters of acyclic alcohols with fatty acids. Concomitantly a portion of the cyclic alcohols generally exist also in a free state. Cotton wax contains both esters of sitosterol ($C_{27}H_{46}O$) and small quantities of both α and β amyrin ($C_{30}H_{50}O$). Wool wax contains both esters of cholesterol and of the triterpene alcohols, lanosterol and agnosterol. Lanosterol (lano-ostandienol) predominates; agnosterol is the corresponding trienol. The sterols are alcohols which possess the cyclic structure of cyclopentanophenanthrene. The constitution, quantitative determination and isolation, and distribution and classification of sterols are fully set forth by BERGMANN in Volume 1 of this series.

Cholesterol, a typical 3-β-hydroxysteroid, is found to the extent of 24–33 per cent in wool wax alcohols, obtained by the saponification of wool wax. Since wool wax yields about 50 per cent of unsaponifiables comprised chiefly of alcohols, the cholesterol content of the wax generally exceeds 11 per cent. The triterpene alcohol content of wool wax alcohols is 19–24 per cent, or over 9 per cent on the wax basis.

The term triterpene alcohol is given to a polycyclic alcohol which has 30 carbon atoms. It may have either four adjoining hexagonal rings, or a steroid structure of three hexagonal rings and one pentagonal ring with three additional methyl groups attached to the ring formation. The extensive studies made of the formula of lanosterol (lanostadienol) by VOSER, MIJOVIC, JEGER, and RUZICKA, which were completed in 1951, reveal that lanosterol is 4,4,14-trimethyl zymosterol, and that it has the skeletal structure given below, with methyl groups other than those regularly found in C_{27} sterols as here indicated.



Lanosterol

1. *Determination of triterpene alcohols.* The cholesterol content of wool wax is easily determined by the well-known Windaus gravimetric method in which cholesterol is precipitated by digitionin to produce the adduct cholesteryl digitonide. LUDDY, TURNER, and SCANLON (1953) reported that the colorimetric

methods previously used for the determination of cholesterol and triterpene alcohols in wool wax did not take into account the overlapping of the absorption bands of the coloured derivatives. They found that at the wavelengths selected, a sample of suitable concentration for the determination of cholesterol was much too concentrated to be used for the triterpene alcohols. They accordingly modified the application of the Liebermann-Burchard reaction by the addition of 1,4-dioxane to the reagent, thus reducing rate of colour development, and the procedure could be employed at room temperature instead of 12°C or below.

By substituting a spectrophotometer for the colorimeter and determining the triterpenes at the secondary maximum at 550 mμ and cholesterol at 630 mμ, a single solution may be used for both. Corrections are made for overlapping absorption bands in both cases. The improved method has been modified by these investigators to make it applicable to the unsaponified wool wax esters, to the mixture of free alcohols obtained by the saponification of wool wax, or to the precipitated cholesteryl digitonide.

2. *Amyrins and lupeol.* Amyrins and lupeol (M.pt. 214°C) are not uncommon constituents of lipid waxes obtained from the bark, leaves, and flowers of plants. These resinols have the empirical formula $C_{30}H_{50}O$, and have a triterpenoid structure, i.e. a skeleton of five adjoining rings. In the case of the amyrins all five rings are hexagonal, whereas in lupeol four are hexagonal and the fifth (*E* ring) pentagonal. Amyrins and lupeol have higher melting points than the sterols and are dextrorotatory. α-amyrin (α-amyrenol) crystallizes in fine, long white needles, and β-amyrin (β-amyrenol) in long hard needles. Their melting point and optical rotation values, and those of the formic and acetic derivatives, and eutectics (crystallized from ethanol) are given in Table 4. β-amyrin occurs in balatas in the form of its acetate.

Table 4. Properties of amyrins

	M.pt. (°C)	*Rotatory power* $[\alpha]_D$ (*in* $CHCl_3$)
α-amyrin	185–187[a] [b]	82·3–82·8,[b] 91·6*B*,[b] 91·4*B*[a]
β-amyrin	197·3,[b] 198–199[c]	87·8–88·4,[b] 88·6[c]
α-amyrin formate	190	80·4
α-amyrin acetate	224–225,[b] 225–226[a]	75·6–76,[b] 77·9, 83·3[a]
β-amyrin formate	210	49·8
β-amyrin acetate	240·5,[b] 238–240[d]	75[d], 81·1–81·4[b]
	Eutectic point (°C)	*Percentage α-form*
Mixture of amyrins	181	77·0
Mixture of amyrin formates	176	71·0

[a] Ruzicka and Wirz (1939); [b] Cohen (1910); [c] Ruzicka and Schellenberg (1937); [d] Lardelli and Jeger (1948); *B* refers to $[\alpha]_D$ in benzene.

III. The Acids of Lipid Waxes

A. Physical properties of fatty acids

Table 5. Melting points and densities of saturated fatty acids

Carbon number	*Geneva name*	*Common name*	*M.pt.* (°C)[a]	*Density* (d_4^{100})
C_{10}	*n*-decanoic	capric	31·1	0·8372[b]
C_{12}	*n*-dodecanoic	lauric	43·8	0·8332[b]
C_{14}	*n*-tetradecanoic	myristic	54·0	0·8306[b]
C_{16}	*n*-hexadecanoic	palmitic	62·7	0·8286[d]
C_{18}	*n*-octadecanoic	stearic	69·5	0·8270[d]
C_{20}	*n*-eicosanoic	arachidic	76·2	0·8240[c]
C_{22}	*n*-docosanoic	behenic	80·4	0·8221[c]
C_{24}	*n*-tetracosanoic	lignoceric	83·7	0·8207[c]
C_{26}	*n*-hexacosanoic	cerotic	88·0	0·8198[c]
C_{28}	*n*-octacosanoic	montanic	90·4	0·8191[c]
C_{30}	*n*-tricontanoic	*n*-melissic	93·1	0·8185[d]
C_{32}	*n*-dotriacontanoic	lacceroic	96·1	0·8180[d]
C_{34}	*n*-tetratriacontanoic	geddic	97·3	0·8176[d]

[a] The melting points are the average of several values reported in the chemical literature; [b] experimental values of Dorinson *et al.* (1942) at 80°C extrapolated; [c] values by Bleyberg and Ulrich (1931); [d] computed values.

B. Separation of n-acids by methylation

Murray and Schoenfeld (1953) were able to determine the relative proportions of the *n*-aliphatic acids in carnauba wax by fractional distillation of their methyl esters. The individual methyl esters were identified through their melting points, solidification points, and crystal spacings, which agreed well with those of Francis *et al.* (1937) for the corresponding methyl esters of synthetic acids having the same number of carbons. The proportion of *n*-acids among acids of carnauba wax has been found by chromatographic separation to be 38 per cent.

Table 6. Properties of carnauba-wax acids

Acid	*Peak temp. distillation methyl esters at 1·00 mm*	*M.pt.* (°C)	*Solidification point* (°C)	*Crystal spacings*	
				$d(001)_B$	$d(001)_O$
n-octadecanoic	155	68·2–68·3	69·2	44·2	40·2
n-eicosanoic	175	75	74·9	49·0	44·2
n-docosanoic	183	79·9	79·6	53·0	48·4
n-tetracosanoic	210	81·1	83·8	58·2	52·8
n-hexacosanoic	225	87·5–87·6	87·2	62·3	56·5
n-octacosanoic	240	90·5	90·4	—	61·3
n-triacontanoic	253	93·1	93·2	72·3	66·1

Table 6 (continued)

Fatty-acid composition of methyl esters

Below C_{18}	4%	Below C_{26}	12%
C_{18}	3%	C_{28}	16·5%
C_{20}	11·5%	C_{30}	7%
C_{22}	9%	C_{32}	none distilled
C_{24}	30%	deficit	7%

C. Iso acids and ante-iso acids

In WEITKAMP's investigations (1945), thirty-two acid components were isolated from the sterol esters separated from a lot of "centrifuged degras." In the separation of four homologous series of acids, WEITKAMP employed a laboratory vacuum still identical with that described by WEITKAMP and BRUNSTRUM (1941), except for the substitution of a more efficient spiral-conical pattern of wire-gauze packing. The packed section was 44 in. in length by 1 in. in diameter. The overall efficiency was approximately 100 theoretical plates. The first series consisted of normal acids with the structure:

$$CH_3—(CH_2)_{2n}—COOH \qquad n = 4 \text{ to } 12 \text{ inclusive.}$$

The second series consisted of iso acids; these have odd-number carbon chains and branching methyl group in the penultimate position:

$$\begin{array}{l} CH_3—CH_2—(CH_2)_{2n}—COOH \\ \quad\quad\quad\

Table 7. Crystal spacings of the iso acids from wool grease

	Long spacing (Å)	Observed side spacings (Å)		
		D_1	D_2	D_3
12-methyltridecanoic . .	26·8	4·39	—	2·33
14-methylpentadecanoic . .	30·7	4·66	3·76	2·32
16-methylheptadecanoic . .	33·8	4·82	3·78	2·33
17-methyloctadecanoic . .	36·2	—	—	—
18-methylnonadecanoic . .	37·8	4·66	3·74	2·33
20-methylheneicosanoic . .	41·4	—	—	2·33
24-methylpentacosanoic . .	48·4	4·60	3·78	2·33

Long spacings of the amides of the iso acids are substantially the same as those given for the acids.

Table 8. Long crystal spacings of some ante-iso acids and corresponding amides

	Long spacings (Å)	
	Amide	*Acid*
d-6-methyloctanoic	19·2	—
d-8-methyldecanoic	23·2	—
d-10-methyldodecanoic . . .	25·9	—
d-12-methyltetradecanoic . . .	29·2	—
d-14-methylhexadecanoic . . .	32·8	33·4
d-16-methyloctadecanoic . . .	—	34·2
dl-16-methyloctadecanoic . . .	35·8	28·0

The results of these studies indicate that the long crystal spacings of the iso series increase linearly with carbon content. The absolute magnitudes of the spacings are greater than the length of a single molecule and somewhat less than two molecules. This indicates the existence of a typical double-layer structure in which the molecules are tilted toward the basal planes. The average increment between members is 3·60 Å.

D. Hydroxy acids

Aliphatic monohydroxy acids are ordinary constituents of the lipid waxes, dihydroxy acids are occasionally encountered, and trihydroxy acids are a rarity.

1. *Saturated monohydroxy acids.* Most of the hydroxy acids recovered on hydrolysis of waxes are either in the α or the ω form. In the plant, the terminal OH group of one molecule tends to esterify the carboxyl of a second molecule to form a polymer, known as an etholide.

There is reason to believe that γ-hydroxy acids exist in the unhydrolysed and unmelted wax substances of plant material. The γ-hydroxy acids readily form lactones when subjected to heat, and such lactones are found in waxes

which have been subjected to processing. The δ-hydroxy acids are the most unstable, and can only be isolated as lactones. Rarely is the OH in the β—or other position than those stated above. This does not hold true for unsaturated hydroxy acids.

Waxes obtained from narrow leaf trees most often contain ω-hydroxy acids. Juniperic (ω-hydroxypalmitic) acid is a common constituent of conifer waxes. Toyama and Hirai (1951) report the presence of 15 per cent of hydroxypalmitic acid (α form) in beeswax as calculated from its acetyl value. They state that ethanol of 55 per cent strength extracts hydroxypalmitic acid with the least amount of other fatty acids and unsaponifiable substance from the hydrolysed wax. Waxes of arid plants usually have the hydroxy acids in the α form and/or lactones of other forms.

There is a wide spread between the melting points of the hydroxy acids and the corresponding normal acids as is shown in Tables 9 and 10.

2. *Dihydroxy acids.* The dihydroxy acids are of interest in helping to identify the monoethenoid acids occasionally encountered in lipid waxes. They are mild oxidation derivatives obtainable by treating monoethenoid acids with alkaline permanganate. For example, the liquid wax (jojoba oil) obtainable from the seed of *Simmondsia californica* Nutt., according to Green, Hilditch, and Stainsby (1936) has as its chief acid, $\Delta^{11,12}$ eicosanoic acid, and a minor amount of docosenoic acid. These monoethenoid acids are readily converted to the corresponding oxidation products, $\Delta^{11,12}$-dihydroxyeicosanoic acid and dihydroxydocosanoic acid.

E. Lactones and Lactides

Lactones are uncommon constituents of unhydrolysed lipid waxes, that is, waxes as they normally occur in plant life. In some instances, lactones are formed as condensation derivatives when the lipid wax is subjected to the pyrolysis which sometimes takes place in melting. Carnauba wax is believed to have about 5 per cent of lactones and lactides. Findley and Brown (1953) report the presence of 2 mol per cent of lactides in the total wax acids, and 54 mol per cent of dihydroxy acids.

1. *δ-lactone in wool wax.* Although the presence of a lactone in wool wax had been reported (erroneously as a lactone of α-hydroxy acid) more than a score of years ago, it remained for Radell, Eisner, and Donahue (1954) to determine its nature with any degree of certainty. These investigators reported (1954) that by the saponification of wool wax and the isolation of the acid fraction of all alkali-soluble material, it has been possible to separate chromatographically from the acid fraction a nonacidic material which appears to be a lactone of molecular formula $C_{20}H_{36}O_2$ representing 12 per cent of the acid fraction. Since the acid fraction amounts to 52·2 per cent, the lactone in the wax must be present to the extent of 6·3 per cent, which is a discovery of considerable importance in elucidating the very complex chemical composition of wool wax.

Infra-red examination of the lactone fraction (of wool wax acids) dissolved

Table 9. Melting points of saturated and hydroxy acids

Carbon number	*Normal acid (°C)*	*α-Hydroxy acid (°C)*	*ω-Hydroxy acid (°C)*
C_{12}	43·8	74	84·5
C_{14}	54·0	81·5	91·3
C_{16}	62·7	86·8	92·4
C_{18}	69·5	91·5	96·9
C_{20}	76·2	94	97·6
C_{22}	80·4	96·5	99·6
C_{24}	83·7	98·5	—
C_{26}	88	99·5	—
C_{28}	90·4	101	—
C_{30}	93·1	102·5	—
C_{32}	96·1	104	—
C_{34}	97·3	104·5 (C form)	—

Table 10. Melting points of C_{16}—C_{18} hydroxy and dihydroxy acids

(α-, β-, γ-, and ω-hydroxy acids and various dihydroxy acids)

Total carbons	*Monohydroxy acids* $C_nH_{2n}(OH)\cdot COOH$	*M.pt. (°C)*	*Dihydroxy acids* $C_nH_{2n-1}(OH)_2\cdot COOH$	*M.pt. (°C)*
(16)	2-hydroxypalmitic	86·5–87 (f.p.)[a, f]	2,3-dihydroxypalmitic	126[k]
	3-hydroxypalmitic	83–83·5[b]	3,12-dihydroxypalmitic	83·4[k]
	4-hydroxypalmitic	79–79·6[c]	7,8-dihydroxypalmitic	115[k]
	16-hydroxypalmitic (juniperic)	93·4,[d] 91[e]	7,16-dihydroxypalmitic	83–84[l]
			threo-9,10-dihydroxypalmitic	86·5[m]
			erythreo-9,10-dihydroxypalmitic	124·3[m]
(18)	2-hydroxystearic	91–92[a]	2,3-dihydroxystearic	125·5–126[n]
	3-hydroxystearic	85,[g] 89[b]	threo-6,7-dihydroxystearic	114
	4-hydroxystearic	79–79·5[c]	erythreo-6,7-dihydroxystearic	122[o]
	18-hydroxystearic	96·6–97·2[d]	threo-7,8-dihydroxystearic	95–96
			erythreo-7,8-dihydroxystearic	132–133[p]
(20)	2-hydroxyarachidic	94, 91–92[j]	threo-9,10-dihydroxyeicosanoic	97–98[q]
	3-hydroxyarachidic	92[k]	erythreo-9,10-dihydroxyeicosanoic (dihydroxygadoleinic)	130·1[m]
	4-hydroxyarachidic	54–55 (lactone)[k]	threo-11,12-dihydroxyeicosanoic	94–95[r]
	20-hydroxyarachidic	97·4–97·6[d]	erythreo-11,12-dihydroxyeicosanoic	130·5[s]

[a] Freezing point by LE SUEUR (1905); [b] melting point by ROBINSON (1934); [c] by HOUSTON (1947); [d] values of five investigators averaged; [e] by HUNSDIECKER and ERLBACH (1947); [f] adapted by RALSTON (1948); [g] by RADCLIFF and GIBSON (1923); [h] by PENZIO (1904); [i] by CHUIT and HAUSSER (1929); [j] by BACZEWSKI (1896); [k] adapted by WARTH (1947); [l] by KERSCHBAUM (1927); [m] values of four investigators averaged; [n] values of three investigators averaged; [o] adapted by MARKLEY (1947); [p] by HUBER (1951); [q] by BROUGHTON (1952); [r] by NIEMANN and WAGNER (1942); [s] by GREEN, HILDITCH, and STAINSBY (1936).

in carbon disulphide showed a carbonyl absorption band of 1738 kaysers (wave number, cm^{-1}) which agrees well with a literature value of 1740 kaysers for a δ-lactone carbonyl. These investigators state that the spectrum of the lactone fraction showed an absorption band near the higher frequency (1770 kaysers) given in the literature for δ-lactones. On the other hand, authentic samples of γ-stearolactone, γ-valerolactone and γ-butyrolactone showed carbonyl bands at 1784, 1788, and 1786 kaysers, respectively.

Refluxing the lactone with methanol and sulphuric acid as catalyst yielded a derivative, presumably a methyl ester, whose infra-red spectrum in carbon disulphide (7·6 g/l) showed an ester carbonyl absorption band at 1744 kaysers, and broad alcoholic hydroxyl bands at 3370 and 3470 kaysers. The presence of a compound such as δ-lactone, would explain the difficulty encountered in obtaining wool-wax acids free from an appreciable saponification number. Furthermore, the data obtained would indicate the presence of hydroxy acid in wool-wax acids. The δ-lactone has a saponification equivalent 311·1, optical rotation of $[\alpha]_D^{20} + 13°$ ($CHCl_3$, $c = 4$), and molecular weight (cryoscopically in benzene) 316.

F. Aromatic acids

The isolation of ferulic acid from the wax of the Douglas-fir bark is indicative that the waxes from the barks of other trees may also contain acids of the aromatic type. These unsaturated aromatic acids are associated structurally with lignin. Caffeic acid (3,4-dihydroxycinnamic acid) is believed to be a constituent of the wax of dry-steamed coffee berries. There is, however, little or no information on the subject in the chemical literature with the exception of the aromatic acid isolated from the bark of the Douglas fir [*Pseudotsuga taxifolia* (Poir.) Britt.]. It is still not known definitely whether the major portion of the ferulic acid is combined with lignoceryl alcohol, or how much if any exists in the free state in the unhydrolysed wax.

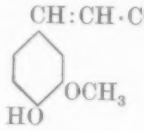
Commercially the wax is solvent-extracted from the waste bark, of which there is a great abundance from the lumbering operations in the northwestern United States. The wax is being produced (1955) on a pilot scale by the Solexol process (a diphasic countercurrent distribution process) to obtain a wax of low resin content. The refined wax has a melting point of 73°C (163°F), is light brown in colour, and very hard. It has a saponification number of 180–210, hydroxyl number 90, and iodine number (Wijs) 12–20. It has a high solvent retention value.

Kurth (1950) reported on the composition of a Douglas-fir wax which had been extracted from waste bark by means of hexane with a yield of 3·6–6·0 per cent. The unsaponifiable substance consisted chiefly of lignoceryl alcohol; it contained a very small amount of phytosterol crystals. The acid fraction of the saponifiables contained 60·5 per cent lignoceric acid and 22 per cent of ferulic acid.

Kurth stated that treatment of a hexane solution of the wax with 5 per cent K_2CO_3 solution extracted both the fatty acid and the aromatic acid. The

largest part of both acids was obtained only after saponification. The composition of the bark wax is different from that obtained from the lignin residue in the hydrolysis of Douglas-fir sawmill residue.

The ferulic acid isolated (M.pt. 168–169°C) when crystallized from alcohol consisted of yellow prisms with a melting point of 170°C. Ferulic acid has the following structural formula.



The chemical composition of refined Douglas-fir wax collected from the data of several investigators is as follows: Glycerides of C_{10}—C_{18} acids, including oleic < 1 per cent; free fatty acids, C_{22}, C_{24}, C_{26} 26 per cent; aromatic acids (as ferulic) free and combined 9–16 per cent; monohydric alcohol esters of wax acids 55–60 per cent, C_{24}—C_{26} esters of C_{22}—C_{26} acids, chiefly the C_{24}, C_{24} ester; phytosterols (M. pt. 134–135°C) free and combined < 1 per cent (α and β sitisterols 0·3 per cent); hydrocarbons XXX 1·7 per cent, phlobaphene and resins > 1 per cent.

IV. The Esters of Lipid Waxes

Although the chemical literature in the past has generally referred to the esters of lipid waxes as compounds of aliphatic alcohols and acids, it is gradually being recognized that aside from the simple esters, there may exist certain amounts of hydroxy esters, acid esters, diesters, and more rarely acid diesters and hydroxydiesters.

Findley and Brown (1953) stated that "it is not enough to know the chemical composition of the hydrolysis products if we are to understand and possibly duplicate the unique physical properties of the waxes. We must also know the extent and manner of combination of the acids and alcohols." These investigators attempt to show how the results of functional group analysis and fractionation by molecular distillation can be utilized in calculating the composition of unhydrolysed lipid waxes. The composition of carnauba wax is computed as: hydrocarbons 12, simple esters 12, hydroxy esters 53, acid esters 5, and diesters 19 mol per cent.

Those waxes which have been fractionally distilled by Findley and Brown (1953) were beeswax, caranda, crude candelilla, refined candelilla, and ouricury. The calculated composition for carnauba wax was compiled from results obtained on the unsaponifiable matter and the acids separated from the soaps by HCl.

For these distillations two still pots were designed and constructed, one 7 cm in diameter and a larger one 12 cm in diameter. The condensing heads could be removed whenever a desired fraction had distilled and replaced with a clean one for further distillation, while the first fraction was weighed and recovered.

Distillations were carried out in the range of 0·1–5 microns pressure depending on the nature of the wax distilled. The fractions were generally removed at 150 ± 10, and 250 ± 10°C in one to several hours time. The residue, as well as the fractions, was weighed.

It is of interest to note that at 150°C all of the hydrocarbons, free alcohols, and free acids of beeswax were distilled, also a portion of the simple esters. At 250°C higher simple esters, and all of the hydroxy esters and acid esters were distilled. The residue carried all of the diesters, acid diesters, and hydroxy diesters.

The acid, ester, and hydroxyl groups, and unsaturated component were determined in moles per kilogram for each fraction. The total of all compositions was calculated from the mean molecular weight as determined by the Rast method of freezing-point depression. Cholesteryl palmitate and behenic acid were used to determine the molecular depression constants.

The composition of unhydrolysed beeswax is now given in moles per cent as hydrocarbons 23, free alcohols 1, free acids 12, esters 45, hydroxy esters 8, acid esters 4, diesters 6, acid esters 1, hydroxy diesters 1, and triester 1.

Carnauba wax is now believed to contain 38–40 per cent of hydroxylated saturated and 14–16 per cent of hydroxylated unsaturated esters by weight. About 30 per cent (wax basis) of the alkyl esters are not hydroxylated. The saturated esters of the higher hydroxy acids furnish the great hardness, and the unsaturated esters of the higher hydroxy acids the lustre, which characterize carnauba wax as an indispensible ingredient for high-grade paste polishes, carbon papers, etc.

V. The Hydrocarbons of Lipid Waxes

It has been postulated that in plant metabolism alcohols with an odd number of carbons (C_9 and upward) are formed first. These alcohols assimilate CO_2 by means of photosynthesis to form fatty acids, and in some instances an O atom is also added to form a hydroxy acid. Alcohols with an even number of carbons become esterified by the acids. Any surplus of alcohols of even number of carbon atoms remains in the free state.

There is always a tendency for the fatty acid to become esterified by an alcohol which has a higher carbon number. Decarboxylation, i.e. loss of a mole of CO_2, takes place only with lower molecular weight wax esters, esters which have about 32 carbon atoms. Decarboxylation always results in a hydrocarbon which has an odd number of carbon atoms, provided the components of the ester have no methyl side chain linkages.

Because of decarboxylation, lower wax esters having alcohol and acid components of equivalent chain lengths, such as cetyl palmitate, or stearyl stearate, are not encountered in waxes of arid plants; but hentriacontane, $C_{31}H_{63}$ (M.pt. 68·2°C) is a common hydrocarbon. It is probably formed from cetyl palmitate by decarboxylation as a last step in the formation of wax on the exterior of the plant itself. The alkanes in lipid waxes, both plant and animal, range from

about 19–35 carbon atoms. The amount of alkanes encountered in lipid waxes ranges from 1–67 per cent, depending on the kind of wax (see Table 11). Candelilla wax has its origin in a plant that thrives in the desert and contains more than 50 per cent of alkanes.

Table 11. Relative amounts and kind of hydrocarbons in lipid waxes

Wax	*% Hydrocarbons*	*Saturated*	*Unsaturated*
Beeswax	10–12	C_{25}—C_{31}	C_{30} (2·5%)
Ghedda	8–9	C_{27}—C_{31}	C_{30} (> 1%)
Chinese insect	2–3	C_{27}	—
Lac	2–6	C_{25}, C_{27}	—
Arjun (Ceroplastes)	3–4	C_{27}—C_{31}	Cycline (cholestene)
Wool	1–2	—	—
Spermaceti	0	—	—
Carnauba	1·5–3	C_{27}—C_{31}	—
Ouricury	7	C_{25}—C_{31} [a]	—
Candelilla	43–59	C_{29}—C_{33} [b]	—
Snow brush	4–7	C_{31}	—
Flax	> 20	C_{29}, C_{31}	—
Cotton	7–9	C_{30}, [c] C_{31}, C_{33}	—
Tea	> 1	C_{29}, C_{31}	—
Sugar cane	3–5	C_{21}—C_{35}	—
Esparto	> 1	C_{31}	—
Apple skin	> 1	C_{27}, C_{29}, C_{30}	—
Orange peel	> 1	C_{29}, C_{31}	—
Cranberry	14	C_{29}, C_{31}	—
Rice bran	< 1	—	Squalene (0·33%)
Olive bark	> 1 [f]	C_{21}—C_{35}	—
Indian corn	< 1	C_{29}, C_{31}	—
Rose	56–57	C_{21}, C_{23}, C_{27} [d]	—
Floral, various	29–67	C_{21}—C_{31} [e]	—

[a] Even-number hydrocarbons, C_{24}, C_{26} isolated and C_{30}—C_{34} indicated by SCHUETTE and KHAN (1953) for ouricury wax. [b] Hydrocarbons (52·2%) consisted of 90% hentriacontane, and 5% each of triacontane and nonacosane, as isolated by SCHUETTE and BALDINUS (1949). [c] Triacontane (C_{30}) was isolated from cotton wax by FARGHER and PROBERT (1923). [d] C_{19}—C_{23}, C_{25}, and C_{29} hydrocarbons have been isolated, in addition to those of an odd number of carbon atoms, by PROPHETE (1926). [e] Wax of pummelo flowers, *Citrus grandis*, contains C_{29} as well as C_{25} and C_{29} hydrocarbons according to YASUNAGA (1952). [f] Most of the bark waxes contain hydrocarbons, but little is known about the amounts or kind; Douglas-fir bark contains 1·7% hydrocarbons, assumably C_{31} and homologues.

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5

THE SYNTHESIS OF PHOSPHOLIPIDS

T. Malkin and T. H. Bevan

CONTENTS

		Page
I.	INTRODUCTION	98
II.	LECITHIN (PHOSPHATIDYL CHOLINE)	100
	Lysolecithin	107
	Glycol lecithins	108
III.	CEPHALIN (PHOSPHATIDYL ETHANOLAMINE)	109
	Lysocephalin	111
	Chimyl and batyl analogues of cephalin	113
	Glycol cephalins	114
	Alkyl β-aminoethyl phosphates	114
IV.	PHOSPHATIDYL SERINE	115
V.	PHOSPHATIDIC ACIDS	116
	1- and 2-Acyl batyl phosphates	118
	Glycol analogues of phosphatidic acids	118
	Higher alkyl dihydrogen phosphates	119
	Bisphosphatidic acids	119
	Bis-glycol phosphatidic acids	120
	Dialkylhydrogen phosphates	120
VI.	PLASMALOGENS	121
VII.	MISCELLANEOUS RELATED SYNTHESES	124
	L-α-Glyceryl phosphoryl choline	124
	L-α-Glyceryl phosphoryl ethanolamine	125
	Choline phosphate	125
	Ethanolamine phosphate	126
	Serine-3-phosphate	128
	Silver salt of phenyl phosphoryl-N-carbobenzoxy ethanolamine	128
VIII.	SPHINGOLIPIDS	128
	Dihydrosphingosine	129
	Sphingosine	134
	Sphingine	137
	REFERENCES	137

5

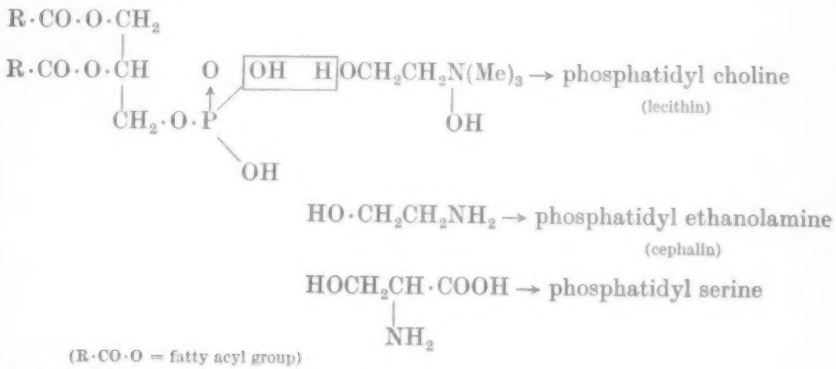
THE SYNTHESIS OF PHOSPHOLIPIDS

T. Malkin and T. H. Bevan

I. Introduction

The phospholipids are fatty substances containing phosphorus and usually nitrogen, and they can be broadly divided into the following four groups.

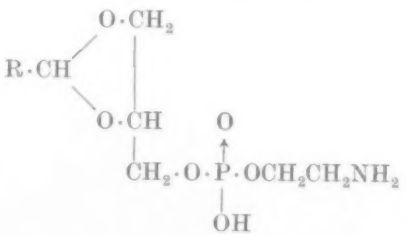
(1) *Derivatives of phosphatidic acids*, I, where the phosphate group is further esterified with choline, ethanolamine, or serine.



Included in this group are the lyso compounds which contain only one fatty acyl radical.

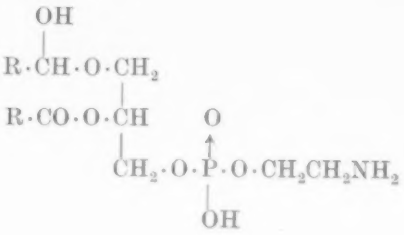
The optical activity of the above compounds and of the glycerophosphoric acid obtained from them by hydrolysis indicates that the phosphate group is in the l position.

(2) *Plasmalogens*, in which a long-chain aldehyde, in the form of an acetal, takes the place of the two fatty-acid radicals in the above phosphatidic-acid derivatives.

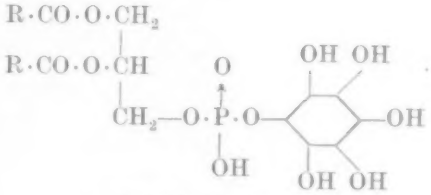


The derivatives with choline and serine probably exist, although they have not been isolated.

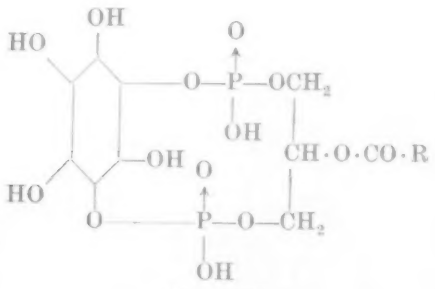
There is still doubt concerning the structure of plasmalogens, and KLENK and DEBUCH,[1] and DEBUCH[1a] have adduced evidence for the following hemiacetal structure:



(3) *The phosphoglyceroinositides* the chemistry of these is not yet fully established and no synthetic work is available. These compounds break down on hydrolysis into fatty acids, glycerophosphoric acid, mesoinositol phosphate and diphosphate, glycerol, sugars, and ethanolamine, all of which may not be found in each case. Two main types appear to exist, based on the following structures:



FAURE and MORELEC COULON[2]
MALKIN and POOLE[3]



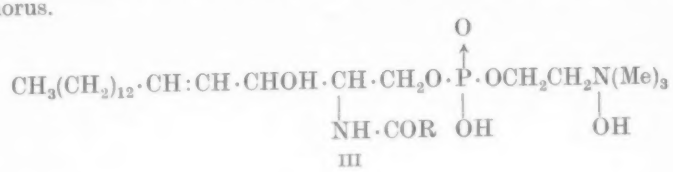
HAWTHORN[4]

(4) *The sphingolipids* are derivatives of the base sphingosine II and the corresponding compound, without the double bond, dihydrosphingosine

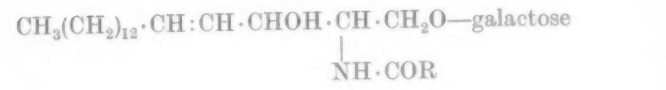
$$CH_3(CH_2)_{12}\cdot CH{=}CH\cdot CH(OH)\cdot CH(NH_2)\cdot CH_2OH$$

II

The chief members of this group are the sphingomyelins III and the cerebrosides IV, although the latter are not phospholipids since they contain no phosphorus.



III



IV

The amidic acids in the above are lignoceric, α-hydroxylignoceric (cerebronic), 9-tetracosenoic (nervonic), and α-hydroxy-nervonic.

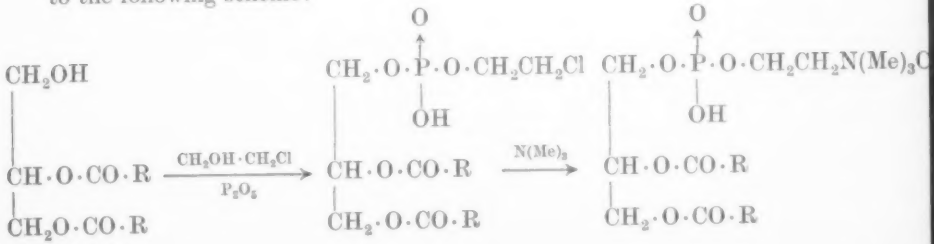
Because of the complexity of the phospholipid mixtures occurring naturally, and the unusual difficulty in their separation, pure individual compounds have only rarely been isolated, and hence synthetic work is of particular importance as a basis of fundamental chemical and biochemical research. It is, therefore, not a little surprising to find that, although the main structural features of many of the above compounds were known during the latter half of the nineteenth century, it is only within the last decade that reliable methods of synthesis have been developed.

At the moment, the position is that satisfactory methods for the synthesis of saturated members of groups (1) and (2) are available, and BAER'S[5] recent synthesis of an unsaturated lecithin for the first time makes it likely that an early expansion into this more important unsaturated field can be anticipated.

In the sphingolipid group, synthetic work has been mainly directed to the synthesis of hydrosphingosine, which has been achieved by a number of workers, and to the synthesis of compounds bearing on its stereochemistry. Two brief accounts of the synthesis of sphingosine have been published, but full details are not yet available.

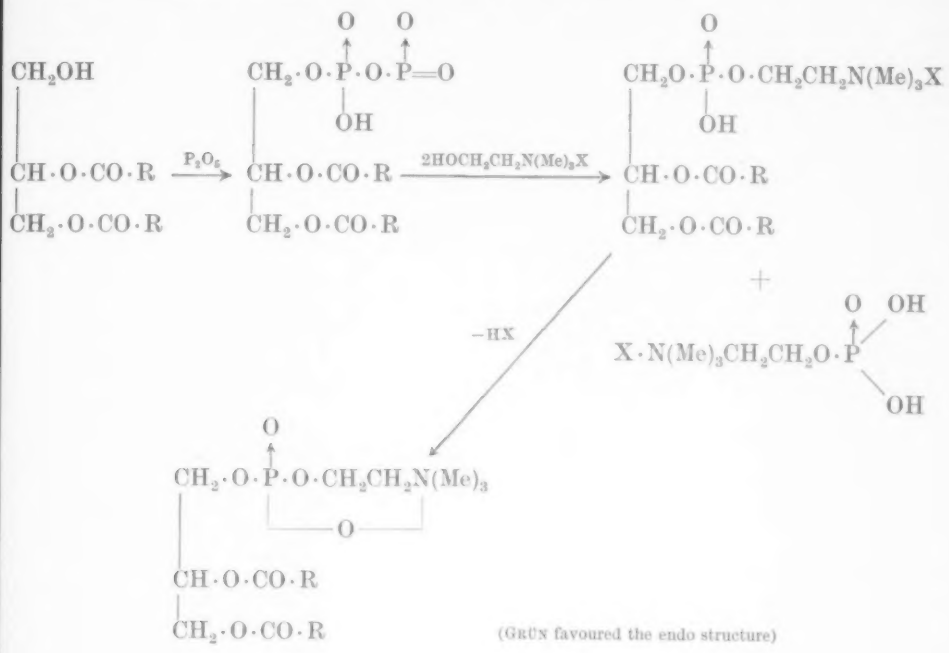
II. LECITHIN (PHOSPHATIDYL CHOLINE)

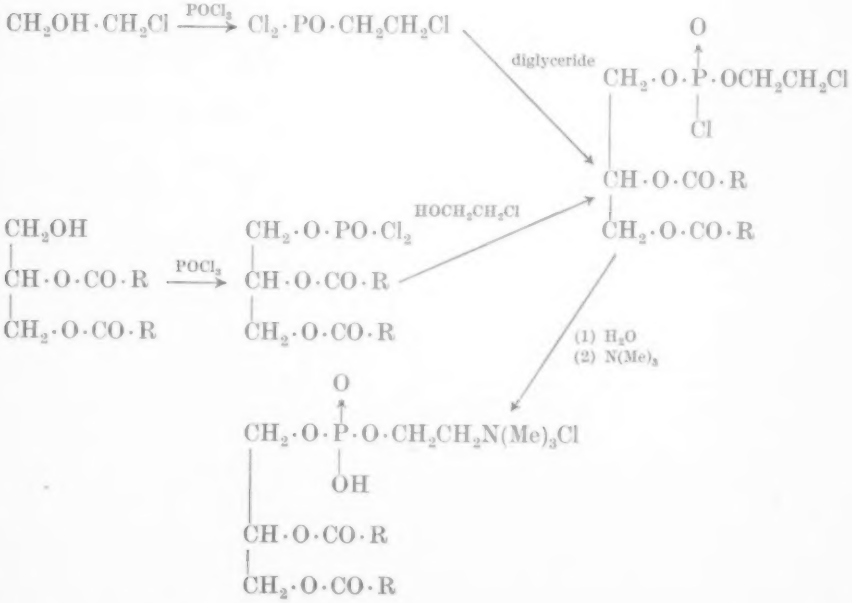
Most of the early work on the synthesis of lecithin was carried out by GRÜN and his co-workers. GRÜN and KADE[6] carried out their first synthesis according to the following scheme:



1:2-distearin

In a later synthesis, GRÜN and LIMPACHER[7] added choline carbonate or acetate to a mixture of 1:2-diglyceride and phosphorus pentoxide.

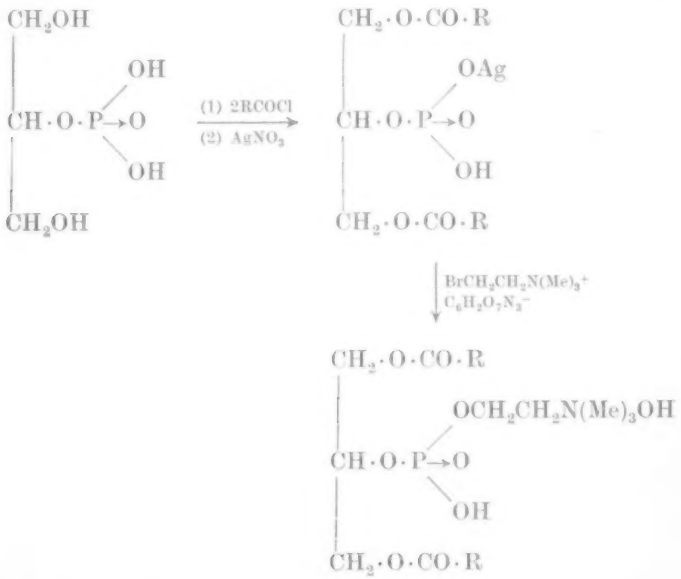




These workers also prepared the 1:3-distearoyl lecithin and, later, a number of mixed acid lecithins.[8] In a third method used by GRÜN,[9] ethylene chlorohydrin or a diglyceride was treated with phosphorus oxychloride, and the product was treated with a diglyceride or with ethylene chlorohydrin respectively, to yield the same final product.

It is doubtful whether GRÜN obtained a pure synthetic product of known constitution. Neither P_2O_5 nor $POCl_3$ are ideal phosphorylating agents, since they give rise to complex by-products (e.g. diglyceryl phosphates). Moreover, at the time of GRÜN's work, there was no satisfactory method for the preparation of 1:2-diglycerides, and it is probable that all the diglycerides used by GRÜN were 1:3-compounds (see Chapter 3, this volume). GRÜN's use of chlorohydrin and trimethylamine is of interest in that their use has been revived in the latest synthesis of BAER,[5] *vide infra*, p. 106.

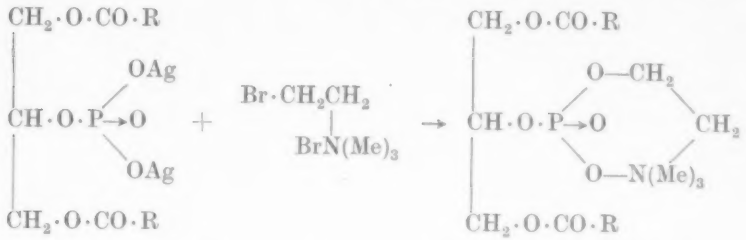
GRÜN's work was followed by that of KABASHIMA, who acylated 2-glycerophosphoric acid with palmitoyl chloride and treated the monosilver salt of the resulting phosphatidic acid with bromocholine picrate.



Neither ROSE[11] nor BEVAN and MALKIN[12] had any success with this method. The acylation, of which details are scanty, but which appears to be a Schotten-Baumen procedure, does not proceed satisfactorily, and the preparation of a monosilver salt is difficult, if not impossible, by the predominant formation of disilver salt.

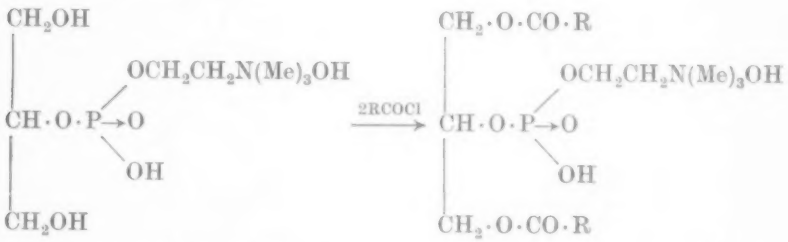
This latter point was confirmed by ARNOLD,[13] who reported a somewhat similar method of preparation. He acylated the sodium salt of 2-glycerophosphoric acid in dry benzene in the presence of pyridine, and converted the product

into the silver salt, which he considered to be mainly the disilver salt. This he refluxed in dry benzene with trimethyl-β-bromethyl ammonium bromide.



Arnold was interested in leprosy therapy, and the acid chlorides he used were prepared from a somewhat crude mixture of chaulmoogric and hydnocarpic acids, and hence his products could not be expected to be pure. Our experience of the final stage of this reaction is that yields are too low for it to be of any practical value.

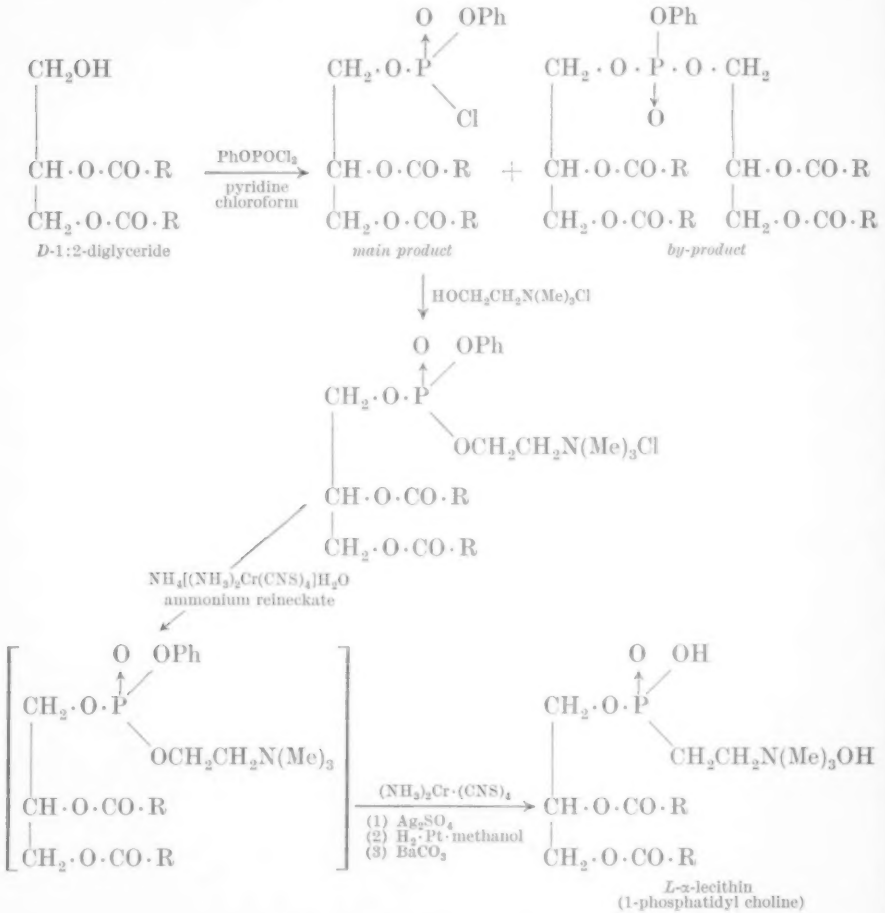
A new approach was that of Obata,[14] who reported a synthesis of lecithin, by the acylation of the choline ester of 2-glycerophosphoric acid with palmityl chloride and pyridine.



The melting point data for the product did not, however, indicate a high degree of purity.

What must be regarded as the first authentic synthesis of lecithin is that of Baer and Kates[15] who prepared a number of *L*- and *D,L*-α-lecithins (1-phosphatidyl choline). The same authors had shown earlier[16] that 1-glyceryl-phosphorylcholine from natural lecithins belongs to the *L* series. Hence, if this compound, which had already been synthesized,[16] could be acylated in the 2·3-positions, lecithins with the configuration of the natural products should result. Various attempts to acylate failed, however, and yielded products which appeared to be mainly mono acylated (lysolecithin). The synthesis was finally effected as follows. *D*-1:2-diglyceride (see p. 73, Chapter 3) was phosphorylated with monophenyl phosphoryl dichloride in the presence of 1 mole of pyridine, giving diacyl *L*-1-glycerylphenylphosphoryl chloride and a by-product, bis-diacylglycerylphenylphosphate. The reaction mixture was immediately treated with choline chloride in the presence of a large excess of pyridine, and the resulting diacyl 1-glycerylphenylphosphorylcholine chloride was isolated and purified by means of its ethyl-acetate-soluble reineckate. The latter was then

converted into the sulphate, and the protective phenyl group was removed by catalytic hydrogenolysis (ADAM'S catalyst), after which the sulphate ion was removed, and the resulting *L*-α-lecithin was crystallized from di-isobutyl ketone. (The change from *D*-diglyceride to *L*-α-lecithin is due to a change in the reference carbon atom.)

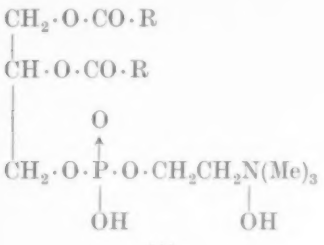


The synthetic *L*-α-dipalmitoyl lecithin and the natural dextrorotatory dipalmitoyl lecithin[17, 18, 19] were found to have the same composition, melting points, solubilities, X-ray diffraction pattern, and optical rotation.

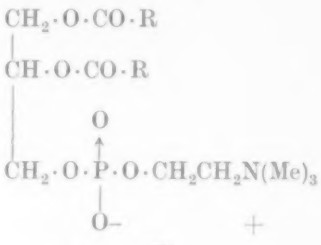
In a later paper, BAER and MAURUKAS[20] described a modification of the above synthesis, which avoided the tedious purification through the reineckate, by successive crystallizations from ethanol and benzene. This modified method was used for the synthesis of *D*-α-dimyristoyl lecithin by BAER and MARTIN.[21]

BEVAN and MALKIN[22] have prepared *DL*-dilauroyl, dimyristoyl, dipalmitoyl, and distearoyl α and β lecithins by the method of BAER and KATES, except that

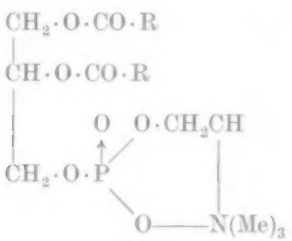
they found it an advantage to use choline iodide instead of the chloride, which is extremely hygroscopic. The *DL*-α and *DL*-β lecithins are very similar to each other and to the *L*-α compounds in melting points and other physical properties and in agreement with the results of BAER and KATES; analytical values correspond to the dihydroxyl structure (A) as distinct from the zwitterion (B) or endo (C) structures.



(A)



(B)



(C)

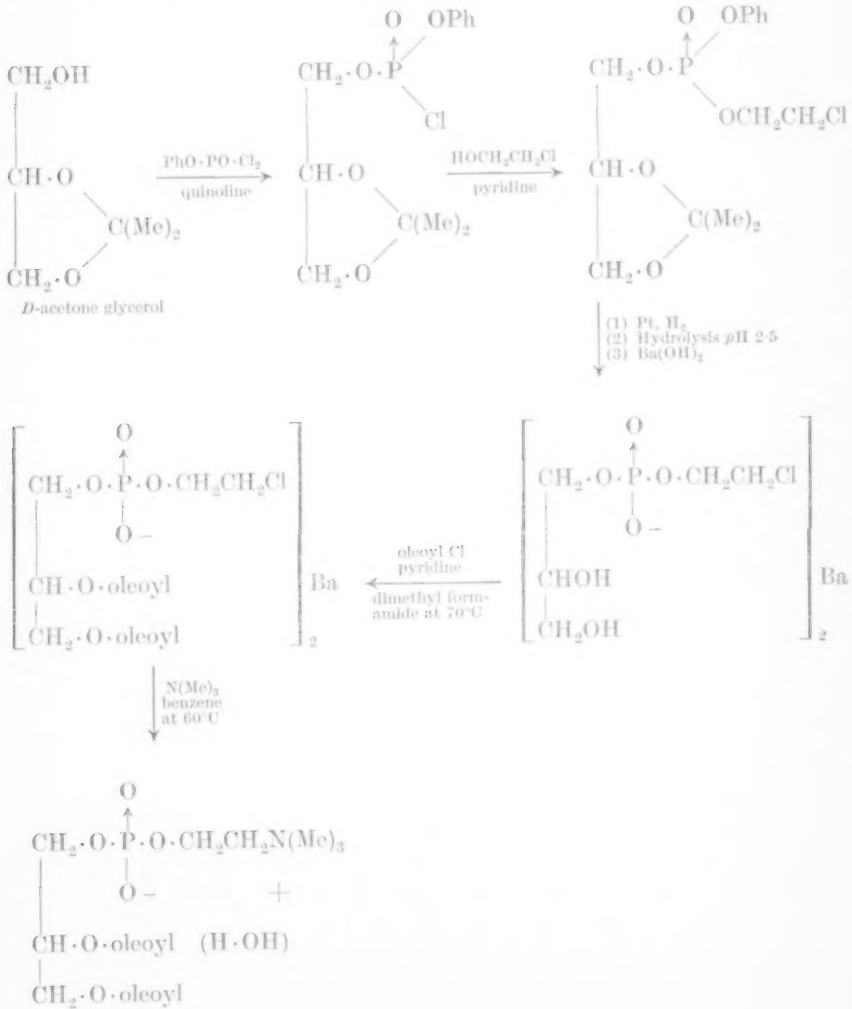
Table 1. M.pts. of synthetic lecithins

BAER *and* KATES		BEVAN *and* MALKIN		BEVAN *and* MALKIN	
L-1:2-distearoyl	230–1·5	*DL*-1:2-distearoyl	231–2	1:3-distearoyl	233–4
L-1:2-dipalmitoyl	234–5	*DL*-1:2-dipalmitoyl	234–5	1:3-dipalmitoyl	236–7
L-1:2-dimyristoyl	237–7·5	*DL*-1:2-dimyristoyl	236–7	1:3-dimyristoyl	239–40
DL-1:2-distearoyl	224–5	*DL*-1:2-dilauroyl	240–1	1:3-dilauroyl	241–2
DL-1:2-dipalmitoyl	227–9				

Sintering is reported for many of the above compounds at varying lower temperatures.

The above synthesis of BAER and KATES can give rise only to lecithins containing saturated acids, because (1) there is no known method for the preparation of unsaturated 1:2 diglycerides, and (2) the removal of the protective phenyl group requires hydrogenolysis which would naturally saturate any unsaturated acid radicals present. Since the majority of natural lecithins are unsaturated, the synthesis of such a compound has been an outstanding problem in this field, and this has now been achieved in the following elegant manner by BAER, BUCHNEA, and NEWCOMBE.[5] *D*-acetone glycerol was treated with phenylphosphoryl dichloride and quinoline, and the resulting *L*-α-glyceryl-phenylphosphoryl chloride was treated with ethylene chlorohydrin in the presence

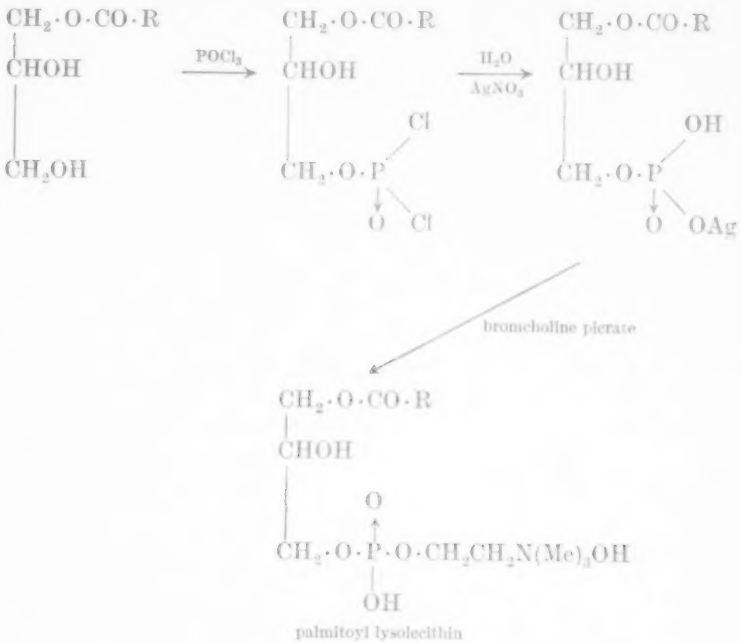
of pyridine. The phenyl group was then removed from the product by hydrogenolysis and the acetone was then removed by mild acid hydrolysis, and the resulting *L*-α-glycerylphosphoryl ethylene chlorohydrin was isolated in the form of its barium salt. This was acylated with oleoyl chloride and pyridine in anhydrous dimethyl formamide, and the dioleoyl compound, on treatment with trimethylamine in benzene at 60°C for four days, gave a mixture of *L*-α-dioleoyl lecithin and oleoyllysolecithin, which could be separated chromatographically on a silicic-acid column.



The special interest in this compound is its solubility in acetone, in which lecithin is usually regarded as being insoluble.

Lysolecithin

KABASHIMA[10, 2nd ref.] claims the synthesis of lysolecithin according to the following scheme.



ARNOLD[13] also claims the synthesis of lysolecithin containing hydnocarpic acid, by the method already described for the synthesis of lecithin, but mono-acylating in the first stage.

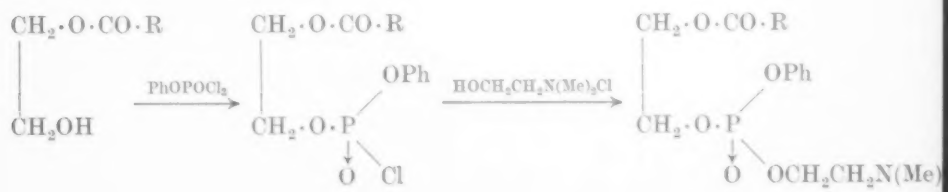
Neither of these compounds can be regarded as approaching purity, although KABASHIMA's product was stated to have haemolytic properties, and presumably, therefore, contained some lyso product.

BAYLIS, BEVAN, and MALKIN[23] and BEVAN and MALKIN[22] have synthesized a series of lysolecithins (C_{12}, C_{14}, C_{16}, C_{18} acids) as follows, starting from the 1-benzyl ether of glycerol.[24]

The position of the phosphate group in the above compound is not yet known with certainty, owing to the possibility of migration to the vacant α-position. Work on the synthesis of this isomer starting from α-acyl β-benzyl glycerol is in progress. Lysolecithins are somewhat hygroscopic and they have not yet been crystallized satisfactorily. They form a normal cadmium-chloride complex with the ratio lysolecithin/Cd $Cl_2 = 2/3$, but the reineckate differs from that of lecithin, in having the composition lysolecithin/reineckate = 2/1, similar to that found by BAER for the following glycol-lecithins. The M.pts. of lysolecithins are not sharp; they sinter in the region of 50–60°C, and give a meniscus at 230–240°C.

Glycol-lecithin

Starting from monoacyl glycols instead of from diglycerides, BAER[25] has synthesized the palmitoyl and stearoyl analogues of lecithin, by the method of BAER and KATES,[15] already outlined,

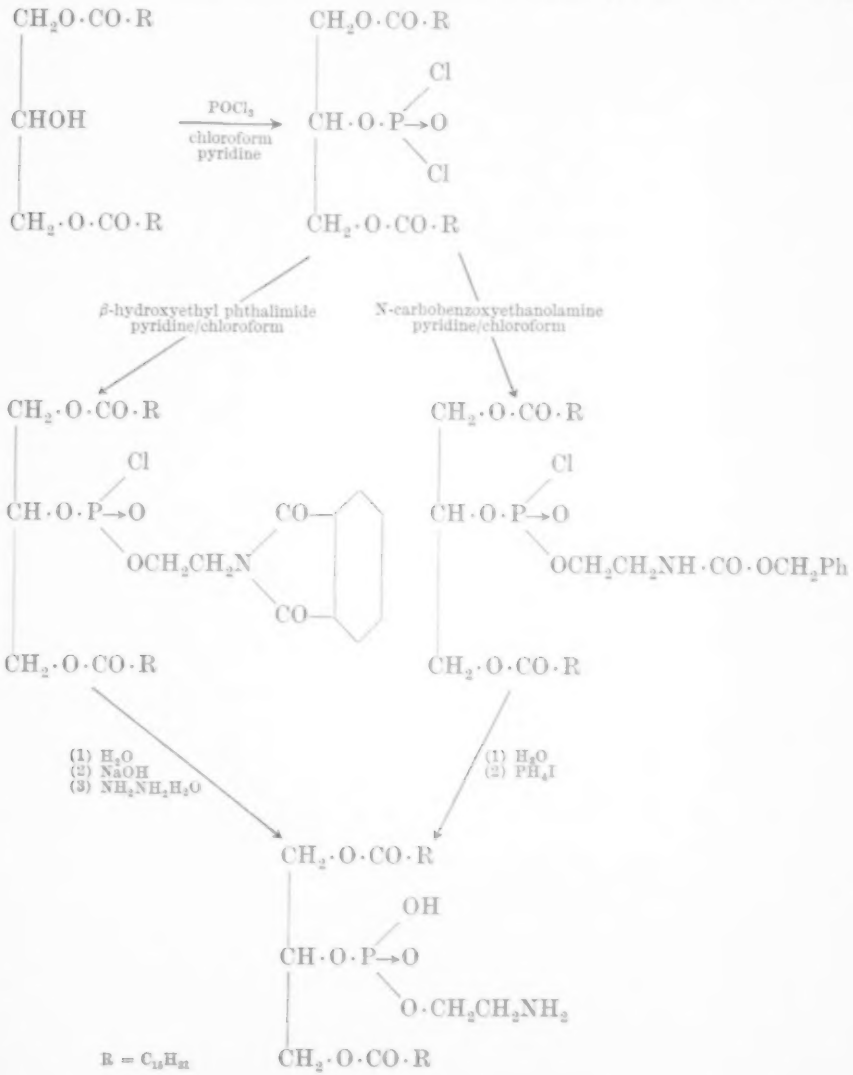


purification through the reineckate, and hydrogenolysis followed in the standard manner. Some slight modifications in technique are necessary in the first stage, because of the greater tendency to form bis (glycol) phosphatidyl phenate,

which reduces the final yield to some 24–30 per cent. As might be expected, these compounds are intermediate in properties between lecithin and lysolecithin. They melt in the same range of temperature (239–243°C) with sintering at a lower temperature. Glycol lecithin is much more soluble in water than the corresponding saturated lecithin and its aqueous solution, like that of lysolecithin, possesses haemolytic properties.

III. Cephalin (Phosphatidyl Ethanolamine)

The early synthetic work on cephalin was carried out on the same lines as that on lecithin by Grün and Limpächer[8] and by Kabashima[10] using



ethanolamine carbonate and β-bromethylamine instead of choline carbonate and bromcholinepicrate respectively, but a critical examination of the work makes it clear that the products were impure mixtures of α- and β-cephalins and/or ethanolamine salts of α- and β-phosphatidic acids.

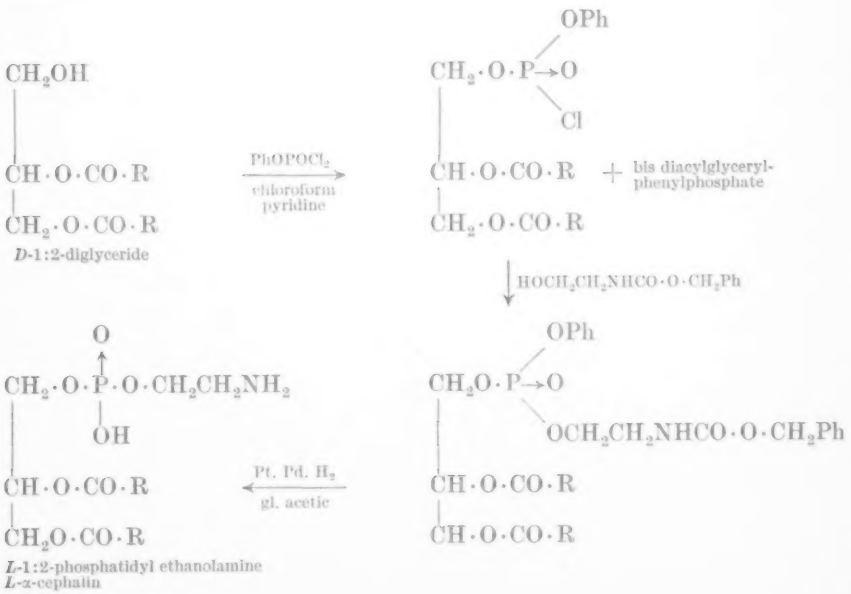
The first authentic synthesis of a cephalin was that due to ROSE[11] who prepared dipalmitoyl β-cephalin (1:3-dipalmitoyl phosphatidyl ethanolamine) according to the scheme on page 109.

1:3-Dipalmitin was treated with phosphorus oxychloride, and the resulting dichloride was treated with ethanolamine in which the amino group was protected by the phthaloyl or the carbobenzoxy group. The latter were finally removed by hydrazine hydrate or phosphonium iodide respectively. ROSE was also able to obtain a small amount of cephalin by KABASHIMA'S method, but he stated that the small yield made the method impracticable.

HUNTER, ROBERTS, and KESTER[26] prepared dimyristoyl- and stearoyl-erucoyl-β-cephalins essentially by the method of ROSE, but dispensing with the tedious separation of the phthalyl intermediate.

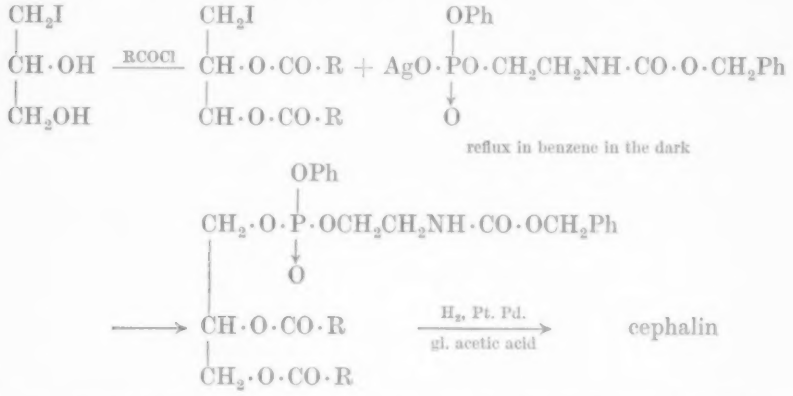
BEVAN and MALKIN,[12] who prepared distearoyl, dipalmitoyl, dimyristoyl, and dilauroyl α- and β-cephalins, found that the HUNTER, ROBERTS, and KESTER modification considerably improved the yield, and that this was also the case if ROSE'S carbobenzoxy method was carried through to the end, without isolation of the intermediate.

A general method for the synthesis of enantiomeric α-cephalins has been reported by BAER, MAURUKAS, and RUSSELL,[27] who synthesized distearoyl, dipalmitoyl, and dimyristoyl cephalins of the *L* series according to the following scheme:



The above synthesis suffers from two disadvantages: (1) the starting material is difficult to prepare and unless carefully handled may change by acyl migration into the 1:3-diglyceride; (2) the first stage leads to considerable amounts of bis-diacyl phosphate as by-product, and this may involve a difficult separation.

In order to avoid these difficulties, BAYLIS, BEVAN, and MALKIN[23] have introduced a new synthesis starting from glycerol-1-iodide which is now readily available (this volume, p. 70). The diacylated glycerol iodide is refluxed with the well-dried silver salt of phenyl phosphoryl-N-carbobenzoxy-ethanolamine, in dry benzene in the dark, and the protecting phenyl and carbobenzoxy groups are then removed by catalytic hydrogenolysis.



This method is superior to any we have used for simplicity, yield, and purity of product.

Table 2. M.pts. of synthetic cephalins

DL-1:2-distearoyl	198[1]			1:3-dimyristoyl	207[1]	173–4[3]
DL-1:2-dipalmitoyl	206[1]			1:3-dilauroyl	210[1]	
DL-1:2-dimyristoyl	207[1]			*L*-1:2-distearoyl	173–175[4]	
DL-1:2-dilauroyl	208[1]			*L*-1:2-dipalmitoyl	172·5–175[4]	
1:3-distearoyl	196[1]			*L*-1:2-dimyristoyl	175–177[4]	
1:3-dipalmitoyl	198[1]	192–3[2]	195–8[2]	1-stearoyl 3-erucoyl	163·5–164[3]	

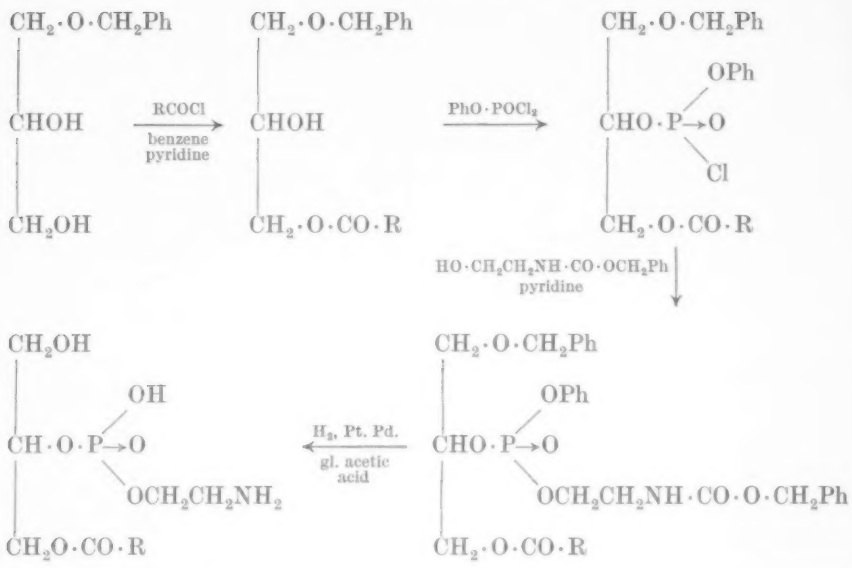
1. BEVAN and MALKIN.[12] 2. ROSE.[11] 3. HUNTER, ROBERTS, and KESTER.[26] 4. BAER, MAURUKAS, and RUSSELL.[27]

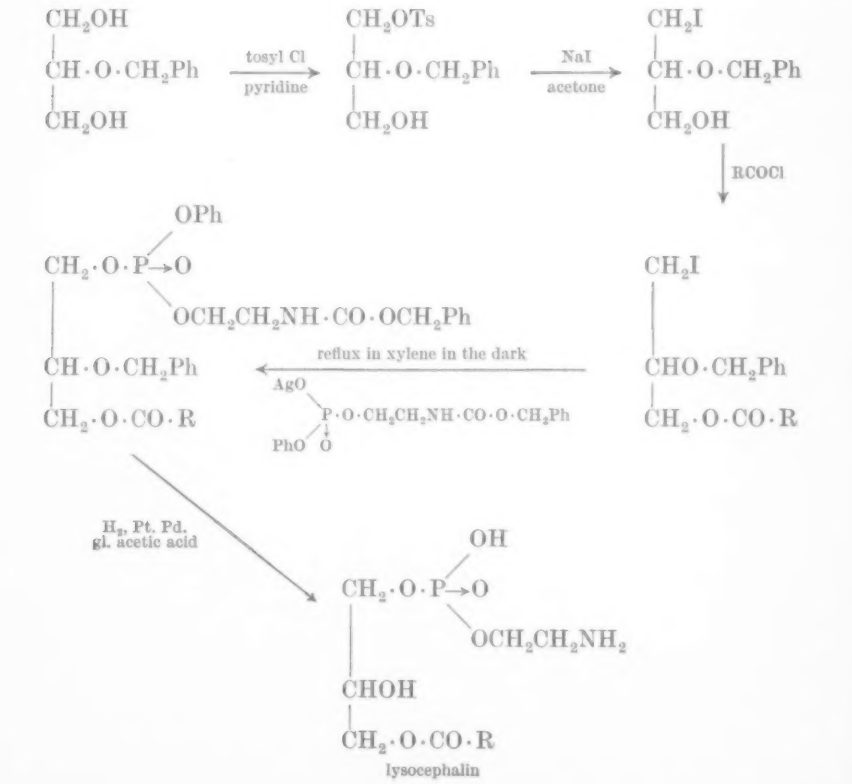
Many of the above compounds are reported to sinter below the M.pt. We find no appreciable sintering when the compounds are pure.

Lysocephalin

This has been synthesized by BAYLIS, BEVAN, and MALKIN[23] according to the scheme at top of page 112.

As is the case with the corresponding lysolecithin, the position of the phosphate group is not definitely established owing to the possibility of migration. The preparation of the isomer has been carried out in our laboratory as shown at foot of page 112.[22]


CH2·O·CH2Ph
CHOH
CH2OH
RCOCl
benzene
pyridine
CH2·O·CH2Ph
CHOH
CH2·O·CO·R
PhO·POCl2
CH2·O·CH2Ph
OPh
CHO·P→O
Cl
CH2·O·CO·R
HO·CH2CH2NH·CO·OCH2Ph
pyridine
CH2·O·CH2Ph
OPh
CHO·P→O
OCH2CH2NH·CO·O·CH2Ph
CH2·O·CO·R
H2, Pt. Pd.
gl. acetic
acid
CH2OH
OH
CH·O·P→O
OCH2CH2NH2
CH2O·CO·R

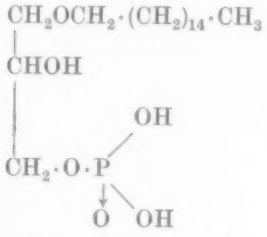

CH2OH
CH·O·CH2Ph
CH2OH
tosyl Cl
pyridine
CH2OTs
CH·O·CH2Ph
CH2OH
NaI
acetone
CH2I
CH·O·CH2Ph
CH2OH
RCOCl
OPh
CH2·O·P→O
OCH2CH2NH·CO·OCH2Ph
CH·O·CH2Ph
CH2·O·CO·R
reflux in xylene in the dark
AgO
P·O·CH2CH2NH·CO·O·CH2Ph
PhO
O
CH2I
CHO·CH2Ph
CH2·O·CO·R
H2, Pt. Pd.
gl. acetic acid
OH
CH2·O·P→O
OCH2CH2NH2
CHOH
CH2·O·CO·R
lysocephalin

The isomeric lysocephalins, with the fatty acyl group in the 2-position, are prepared similarly, starting with glycerol-1-benzyl ether. They are converted into the 3-acyl isomers, by acyl migration, when treated with mineral acids.

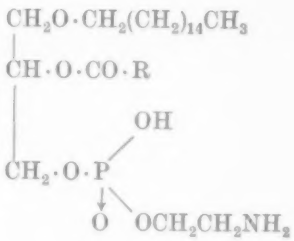
The 2- and 3-acyl lysocephalins (lauroyl → stearoyl) melt in the region of 218–225°C, with sintering at 170–180°C.

Chimyl and batyl analogues of cephalin

In an investigation on plasmalogens, KLENK and DEBUCH[1] isolated a mixture of chimyl and batyl phosphoric acids (*a*) which they considered was derived from a cephalin type of compound (*b*).

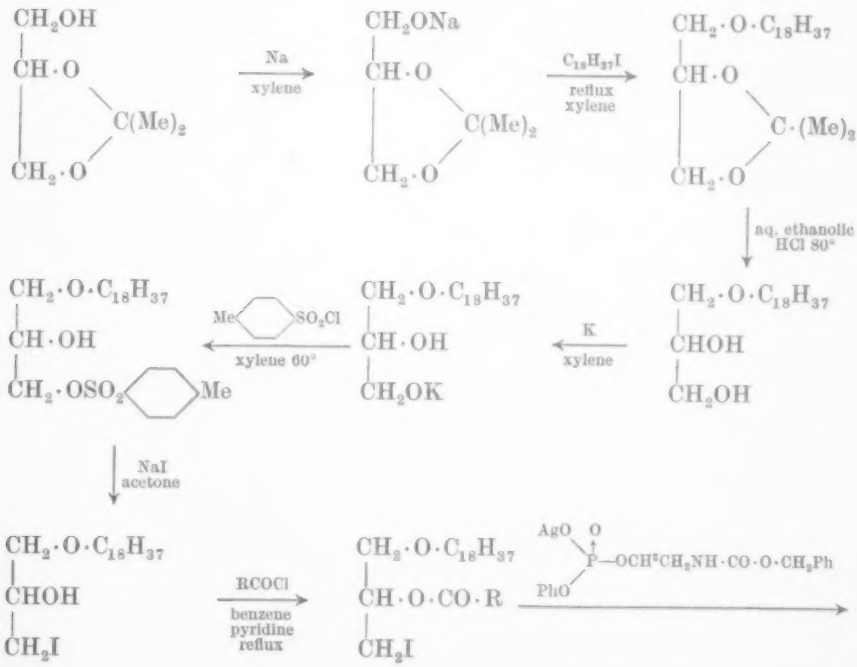


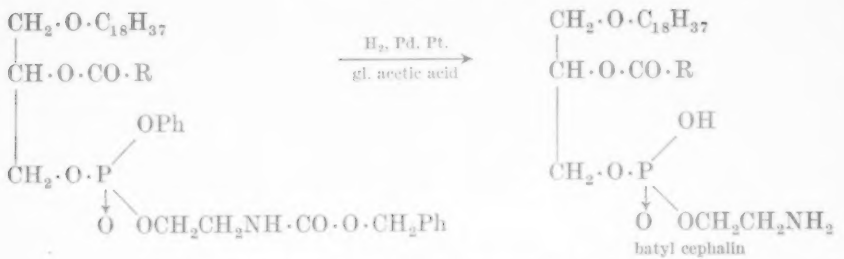
(*a*) chimyl phosphate $(CH_2)_{14}$
batyl phosphate $(CH_2)_{16}$



(*b*) chimyl cephalin

BAYLIS and MALKIN[28] have synthesized a number of chimyl and batyl cephalins as follows:





These compounds are very similar in properties to the cephalins. They are insoluble in boiling ether, soluble in chloroform and glacial acetic acid, and they can be crystallized from methanol-ethanol mixtures.

Table 3. M.pts. of batyl and chimyl cephalins

	Stearoyl	*Palmitoyl*	*Myristoyl*	*Lauroyl*
Batyl . .	195°C	197	200	203
Chimyl . .	198°C	201	205	207

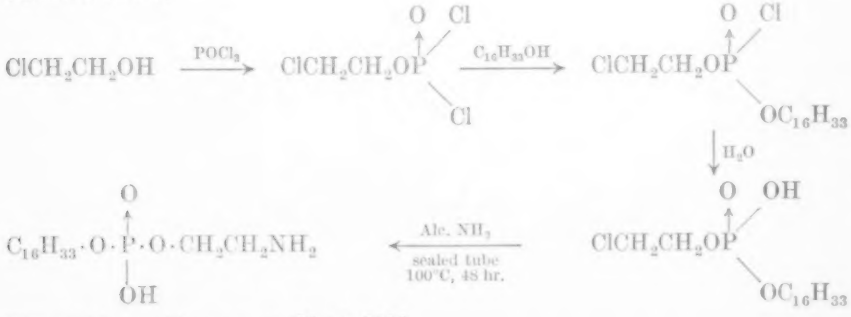
Glycol cephalins

A series of these has been prepared in high yield by BAYLIS, BEVAN, and MALKIN,[23] from the readily accessible acyl glycoliodohydrins.[29] These were refluxed with benzene with stirring, in the dark, with the silver salt of phenyl phosphoryl-N-carbobenzoxyethanolamine giving up to 85 per cent yield of the phenyl carbobenzoxy intermediate. Hydrogenolysis in glacial acetic acid in the presence of a 1/1 mixture of palladium black/ADAM's catalyst gave a 70–80 per cent yield of glycol cephalin. These compounds are conveniently recrystallized from methanol-ethanol mixture.

M.pts.: stearoyl, 216–17°C; palmitoyl, 218–19°; myristoyl, 222–3°; lauroyl, 224–5° (all with decomposition).

Alkyl β-aminoethyl phosphates (alkyl analogues of cephalin)

CHRISTENSEN[30] synthesized hexadecyl β-aminoethyl phosphate by the method outlined below:



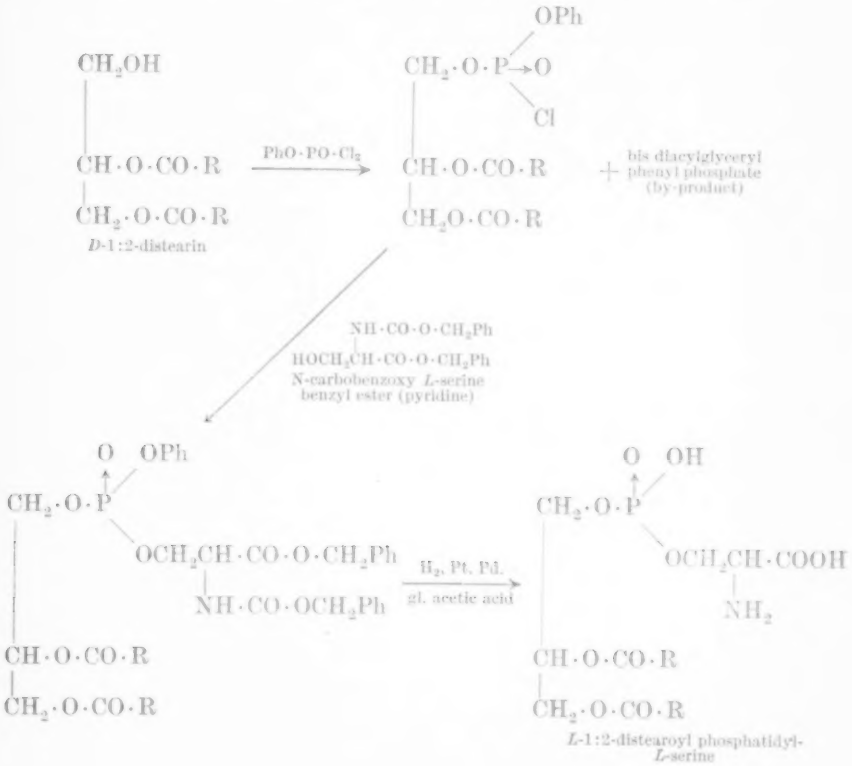
The yield was 18 per cent, M.pt. 226°.

Baylis and Malkin[28] have prepared a series of these compounds in high yield, by refluxing the appropriate alkyl iodide and the silver salt of phenyl phosphoryl-N-carbobenzoxyethanolamine in dry benzene in the dark, followed by the standard hydrogenolysis procedure. The compounds are insoluble in boiling ether, and they are conveniently crystallized from ethanol.

M.pts.: octadecyl, 216°C; hexadecyl, 218°; tetradecyl, 222°; dodecyl, 224°.

IV. Phosphatidyl Serine*

L-1:2-distearoyl phosphatidyl *L*-serine has been synthesized as follows by Baer and Maurukas,[31] and its properties have been found to be identical with those of the reduction product of phosphatidyl serine of ox brain.



Phosphatidyl serine is insoluble in most organic solvents, but it is moderately soluble in chloroform. It is also insoluble in water, but in the presence of alkali it readily forms aqueous emulsions. It melts with decomposition at 159–61°C.

* Bevan and Malkin have recently synthesized phosphatidyl serine by the interaction of distearoyl glycerol iodide and the silver salt of phenyl phosphoryl-N-carbobenzoxy-*D,L*-serine benzylester, followed by hydrogenolysis of the protecting groups.

V. Phosphatidic Acids

The diacyl esters of glycerophosphoric acid were named phosphatidic acids by Channon and Chibnall,[32] who were the first to report their occurrence in nature. Because of their relationship to lecithin and cephalin, they have long attracted attention, and numerous syntheses have been described, by the action of metaphosphoric acid,[33] phosphorus pentoxide[6, 34, 35] or phosphorus oxychloride[36] on diglycerides, or by the acylation of glycerophosphoric acid.[10, 13, 3rd ref.]

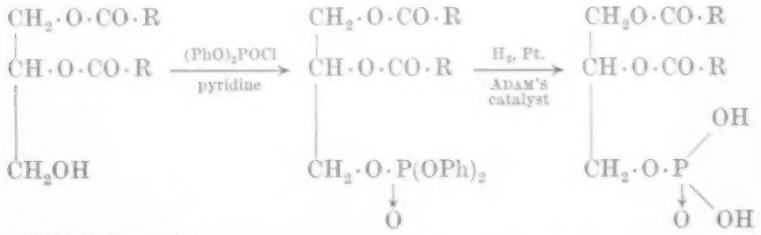
It is, however, extremely doubtful whether any of these earlier workers handled a pure phosphatidic acid, for in addition to the uncertainty surrounding the structure of certain of the diglycerides, and the possibility of the formation of bis-phosphatidic acids, it is now known that phosphatidic acids rapidly lose their fatty acids if exposed to moisture,[37] and also that they combine with cations with avidity to form extremely stable quarter salts, e.g.

$$RPO(OH)_2RPO(OH)(ONa)$$

from which they can be satisfactorily regenerated only by treatment with ion-exchange resins[38] (Amberlite IR.120).

Rose[11] phosphorylated 1:3-dipalmitin with phosphorus oxychloride in chloroform in the presence of quinoline, and isolated the phosphatidic acid as the quarter quinolinium salt.* He claimed to obtain the free phosphatidic acid from this by treatment with hydrochloric acid, but neither Hunter, Roberts, and Kester[26] nor the present authors (unpublished) were able to obtain nitrogen-free products by this method.

Baer[39] phosphorylated a number of *D*-1:2-diglycerides, using diphenylphosphoryl chloride in pyridine, and obtained high yields of the diphenyl esters. Hydrogenolysis of these with Adam's catalyst in glacial acetic acid gave high yields of phosphatidic acids, but their M.pts. were so unreproducible that it was not thought worth while to give them.



R = $C_{17}H_{35}$, $C_{15}H_{31}$, $C_{13}H_{27}$

Baer also made some interesting comparisons between the choline and ethanolamine salts and esters of phosphatidic acids, but in view of Uhlenbroek and Verkade's[38] explanation of his variable M.pts., these might well be reinvestigated.

* The author refers to this salt as the "half" quinolinium salt, but since it contains 2 moles of the dibasic phosphatidic acid to 1 mole of quinoline, it is better described as a quarter salt.

The latter authors prepared a series of 1:2- and 1:3-phosphatidic acid by the same method as BAER, but they used for hydrogenolysis a platinum on charcoal catalyst in dry ethyl acetate or dioxan. They showed that when ADAM's catalyst is used, the small amounts of alkali which it retains forms a quarter salt with the phosphatidic acid, which may lower the M.pt. 10–15°C. The same authors mention the instability of these acids in the presence of moisture, and stress the necessity of keeping them *in vacuo* over P_2O_5.

UHLENBROEK and VERKADE's results have been fully confirmed by BEVAN, BROWN, and MALKIN (unpublished), who found it possible to use ADAM's catalyst for the hydrogenolysis, if it were first acid washed to free it from alkali.[40, 41]

More recently, HESSEL, MORTON, TODD, and VERKADE,[42] and BAYLIS, BEVAN, and MALKIN[30] have prepared phosphatidic acids in good yields from diacylglycerol iodohydrins and silver dibenzyl phosphate and silver diphenyl phosphate respectively. The dibenzyl and diphenyl esters were then submitted to catalytic hydrogenolysis.

X-ray and thermal examination in the Bristol laboratories[43] show that phosphatidic acids exist in two crystalline forms. The two forms of the β acids melt at widely different temperatures, but there is no appreciable difference in the M.pts. of the two forms of α acids.

Table 4. M.pts. of phosphatidic acids and their diphenyl esters

	Acids			*Diphenyl ester*		
	DL-1:2-	*DL*-1:3-	*DL*-1:3-	*DL*-1:2-	*DL*-1:3-	*L*-1:2-[4]
			* Lower M.pt.			
Distearoyl	70–71[1, 2, 3]	68·5–69·5,[1] 69–70[2]	58–59[2]	58–59,[1] 58[3]	50·5–51·5, 51–52[1, 2]	50·5–51·5
Dipalmitoyl	62·5–63[1, 2, 3]	63–64,[1] 64–65[2]	49–50[2]	51–52[1, 2]	43–44[1, 2]	47–48
Dimyristoyl	54–55[2, 3]	55[2]	35–36[2]	44[2]	36–37[2]	38–39
Dilauroyl	44–45[2]	44[2]	11–12[2]	32–33[2]	28–29[2]	
1-Stearoyl-2-palmitoyl[1]	62·5 63·5			53–54		
1-Palmitoyl-2-stearoyl[1]	73·5 74·5			50·5–51·5		
1-Stearoyl-3-palmitoyl[1]		63·5 64·5			39·5 40	

1. UHLENBROEK and VERKADE.[38] 2. BEVAN, BROWN, and MALKIN.[41] 3. HESSEL, MORTON, TODD, and VERKADE.[42] 4. BAER.[39]

1- *and* 2-*acyl batyl phosphates*

A number of batyl analogues of phosphatidic acids have been synthesized from mono-acyl batyl esters by VERKADE and STEGERHOEK[44] and similarly for the 1-acyl esters. The batyl alcohol was prepared in 87 per cent yield by the oxidation of allyl octadecyl ether with performic acid, and the isomeric acyl

esters were prepared by protecting the primary hydroxyl with trityl, acylating the secondary hydroxyl, and finally removing the trityl group by hydrogenolysis, to give the 2-acyl isomer, or by an acid medium to yield the 1-isomer by acyl migration.

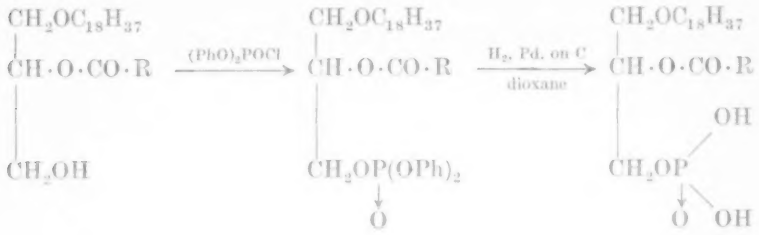


Table 5. M.pts. of acyl butyl phosphates and esters

	Diphenyl esters, M.pt., °C	*Acyl butyl phosphates, M.pt., °C*
1-Stearoyl . .	45·5–46	62–62·5
1-Palmitoyl . .	42·5–43	57·7–58·1
2-Stearoyl . .	48–49	69·8–70·4
2-Palmitoyl . .	46–46·5	65·2–65·9

Glycol analogues of phosphatidic acids. (Acylglycolphosphoric acids.)

Van der Neut, Uhlenbroek, and Verkade[45] have prepared a number of these by the action of diphenylphosphoryl chloride on monoacylglycols, followed by hydrogenolysis.

It was during this work that these authors first noted the formation of quarter sodium salts when Adam's catalyst was used (see p. 117). Acylglycolphosphoric acids crystallize well from anhydrous ethyl acetate or anhydrous acetone. They appear to be more stable than the phosphatidic acids, and they can apparently be kept for long periods without special precautions.

Table 6. M.pts. of acylglycolphosphoric acids and their diphenyl esters

	M.pt., °C	*M.pt., °C*
Behenoyl . .	49–50	96·5–97·5
Stearoyl. . .	38·5–39·5	89·5–90·5
Palmitoyl . .	31·5–32·5	85–86
Myristoyl . .	23·5–24·5	79·5–80·5

Higher alkyl dihydrogen phosphates

BIEHRINGER[46] described a preparation of monohexadecyl dihydrogen phosphate, by the action of phosphorus pentoxide on hexadecanol, a reaction which was later shown by CHEBULIEZ and WENIGER[47] to yield both mono- and di-esters. PLIMMER and BURCH[48] obtained the same two products by the action of phosphoryl chloride on hexadecanol. CHRISTENSEN[30] criticized the latter work, but it is probable that his own preparations were contaminated with the quarter sodium salt of the monoalkyl phosphate, and in general, these earlier methods give rise to products of doubtful purity. BROWN, MALKIN, and MALIPHANT[41] synthesized a series of even-membered phosphates ($C_{12} - C_{18}$) by treating the alcohols with diphenylphosphoryl chloride in the presence of pyridine, and removing the phenyl groups in the product by hydrogenolysis in the presence of ADAM's catalyst, which had been acid washed free from alkali, to avoid formation of quarter sodium salt.

The same diphenyl intermediate may also be made from the alkyl iodide by refluxing with the silver salt of diphenyl phosphate in the dark.[40]

Alkyl dihydrogen phosphates can be crystallized from light petroleum and they are similar in appearance to the fatty acids. They melt roughly 10°C higher than the fatty acid of the same carbon content, viz: octadecyl, 83°; hexadecyl, 75–76°; tetradecyl, 69°; dodecyl, 59·5°. X-ray investigation shows that they crystallize in layers of double molecules, and that they are dimorphous.

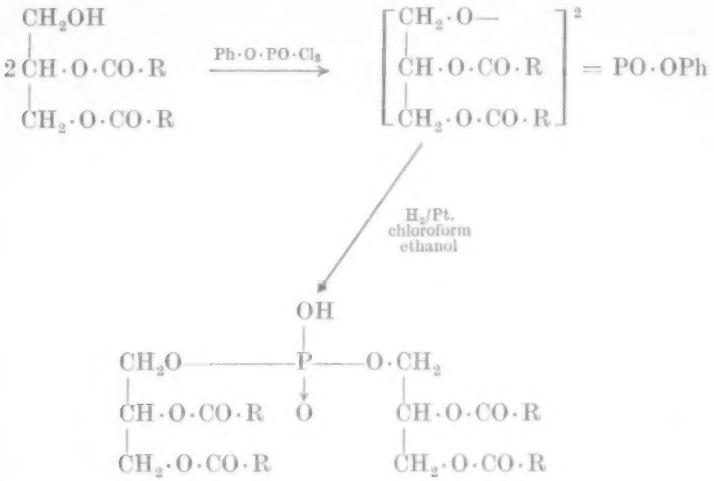
The disodium salts of these alkyl phosphates are difficult to prepare owing to ready hydrolysis to the monosodium salts, and although the latter are reasonably stable they tend to change on crystallization into the extremely stable quarter-sodium salts.[49] These can be prepared by quarter titration of the alkyl phosphates, by crystallizing the sodium-hydrogen salts from acetic acid, or by pouring an alcoholic solution of the alkyl phosphate into aqueous sodium chloride. The quarter salts are freed from cations only with difficulty, and this is best achieved by the use of an ion-exchange resin (Amberlite IR-120).

Bisphosphatidic acids

These compounds are of interest because of their relationship to the cardiolipins, which are probably polyphosphatidic acids, and they may therefore prove useful substitutes for the latter in the serodiagnosis of syphylis. The phenyl ester is formed as a by-product in the BAER and KATES synthesis of lecithin,[15] and if, in the first stage of this reaction, two molecules of diglyceride are used to one of monophenyl phosphoryl dichloride, the bisphosphatidic acid phenyl ester is obtained in almost quantitative yield.[50] Catalytic hydrogenolysis readily removes the phenyl groups giving the free acids, which can be purified

by crystallization from acetone. They are readily soluble in benzene, chloroform, or ether, at room temperature. They are less soluble in methanol, ethanol, dioxane, and acetone, and are insoluble in water.

Tetramyristoyl bis-(*L*-α-glyceryl) phosphoric acid was found to possess cardiolipinlike activity.[51]



M.pts.: bis-L-α-phosphatidic acids

Stearoyl 69·5–70·5° Palmitoyl 62–63° Myristoyl 49–50°.

Bis-glycol phosphatidic acids

In exactly the same manner, but starting from glycol monoesters, Baer[25] has prepared the stearoyl and palmitoyl glycol analogues of bisphosphatidic acids, which were purified by crystallization from 99 per cent ethanol. They are insoluble in cold ether or acetone, very slightly soluble in methanol, ethanol, ethyl acetate, petroleum ether, or benzene, but very easily soluble in chloroform, and very soluble in warm methanol, ethanol, acetone, ethyl acetate, benzene, and petroleum ether.

M.pts. (acids): stearoyl, 92·5–93·5°; palmitoyl, 89–90·5°.

M.pts. (phenyl esters): stearoyl, 59–60°; palmitoyl, 51·5–52·5°.

Dialkyl hydrogen phosphates

In exactly the same manner as the above, but using two moles of higher alcohol to one mole of phenyl phosphoryl dichloride, Brown, Malkin, and Maliphant[41] prepared a series of dialkyl phosphates (even members, $C_{12} - C_{18}$).

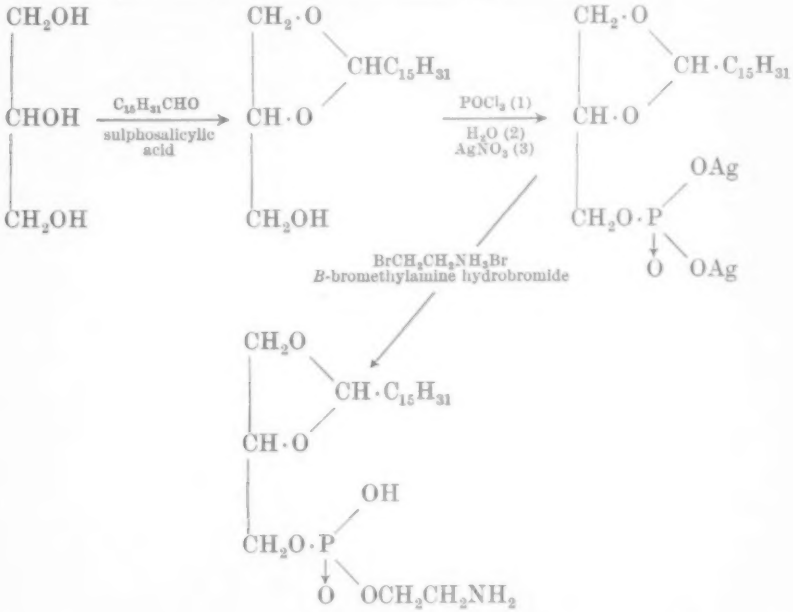
Dialkyl hydrogen phosphates are similar in appearance and close in M.pts. to the monoalkyl dihydrogen phosphates, from which they are readily distinguished however by exhibiting spherulite formation on solidification from the melt.

M.pts.: C_{18}, 82°; C_{16}, 75°; C_{14}, 68°; C_{12}, 59°C.

VI. Plasmalogens

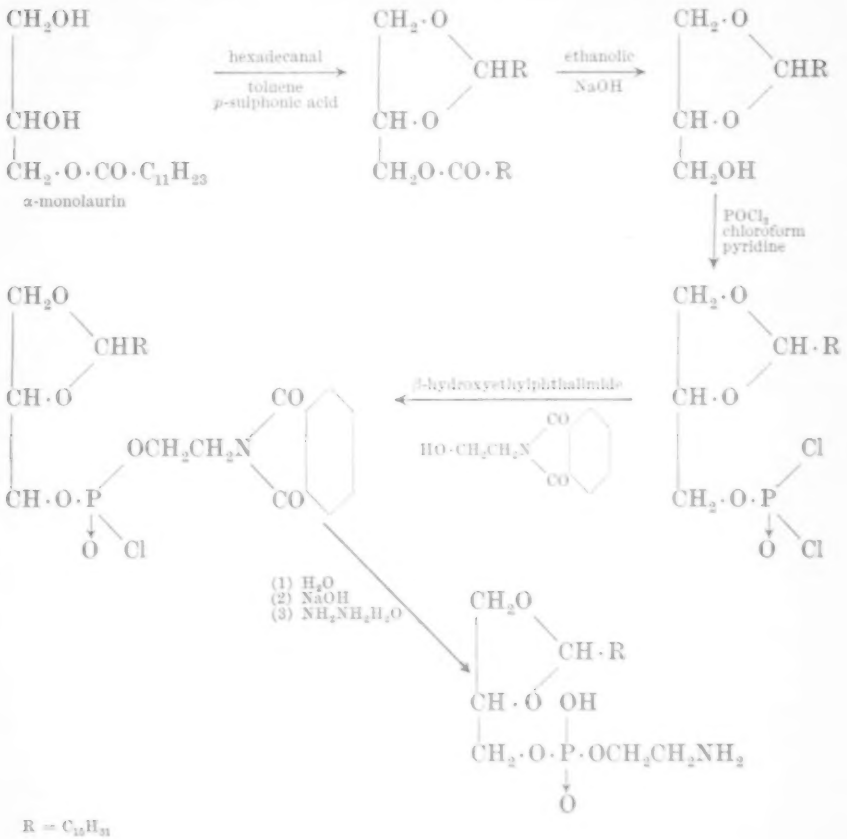
Plasmalogens were discovered in brain and skeletal muscles by Stepp, Feulgen, and Voit,[52] who found that, on treatment with acids or mercuric chloride, they yielded a substance which gave an intense Schiff's reaction, and which was found by Feulgen, Imhauser, and Behrens[53] to be a mixture of aldehydes, mainly hexadecanal and octadecanal. Feulgen and Bersin[54] later showed that plasmalogens were acetal phosphatides, closely related to phosphatidyl ethanolamine, namely, 2-aminoethyl-2:3-O-hexa- or -octa-decylidine-1-glycerophosphate. Analogy with lecithin and cephalin and the optical activity of plasmalogens, reported by Thannhauser, Boncoddo, and Schmidt,[55] indicate the 2:3-acetal structure.

In 1941 Bersin, Moldtmann, Nafziger, Marchand, and Leopold[56] published a synthesis according to the following scheme:



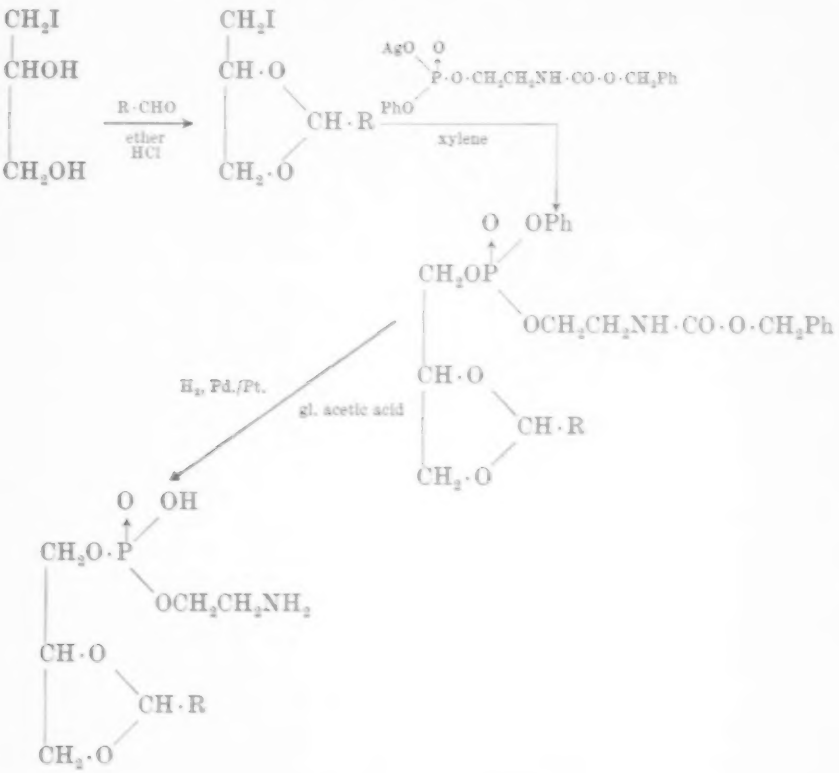
but doubts may be expressed concerning the nature of their final product. Thus, the condensation of glycerol and aldehydes may give rise to cis and trans forms of both 1:2- and 1:3-acetals, *cf.* Hibbert and Carter,[57] and Verkade and van Roon.[58] Moreover, in the analogous synthesis of cephalin, Bevan and Malkin[12] found that the action of 2-bromethylammonium bromide on disilver phosphatidate gave only a trace of the desired product. Bersin *et al.* stated that their product readily hydrolysed into ethanolamine and acetal-glycerophosphoric acid (plasmalogenic acid), for which reason purification by crystallization was not advisable. This, however, is not the normal behaviour of an aminoethyl phosphate, and it is likely that their product was predominantly a hydroxyethylamine salt.

EGERTON and MALKIN[59] carried out a synthesis as follows:



In order to avoid 1:3-acetal formation, hexadecanal was condensed with 1-monolaurin, and the lauryl group was then removed with ethanolic sodium hydroxide, to which the acetal is stable. The acetal was then converted by phosphorus oxychloride into plasmalogenoyl dichloride, which was condensed with 2′-hydroxyethylphthalimide. Removal of the phthaloyl group with aqueous hydrazine yielded a crystalline product, which gave a positive Feulgen-Schiffs aldehyde test and a positive ninhydrin test.

An improved synthesis, according to the following scheme, has been announced by MALKIN and WEBLEY:[60]



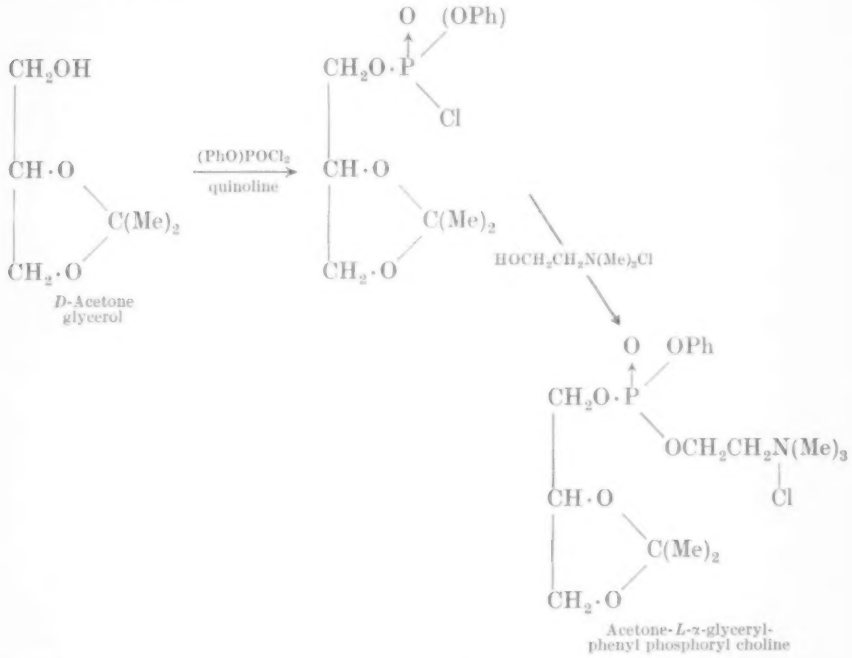
Glycerol iodohydrin is condensed with the aldehyde in dry ether, containing a trace of hydrogen-chloride gas, and the product is refluxed in xylene, with stirring, in the dark, with the silver salt of phenyl-N-carbobenzoxy-ethanolamine. Hydrogenolysis gives the required product in high yield.

M.pts. of plasmalogens. 2:3-octadecylidine, 224°; 2:3-hexadecylidine, 227°; 2:3-tetradecylidine, 230°; 2:3-dodecylidine, 233°.

VII. Miscellaneous Related Syntheses

L-α-glyceryl phosphoryl choline

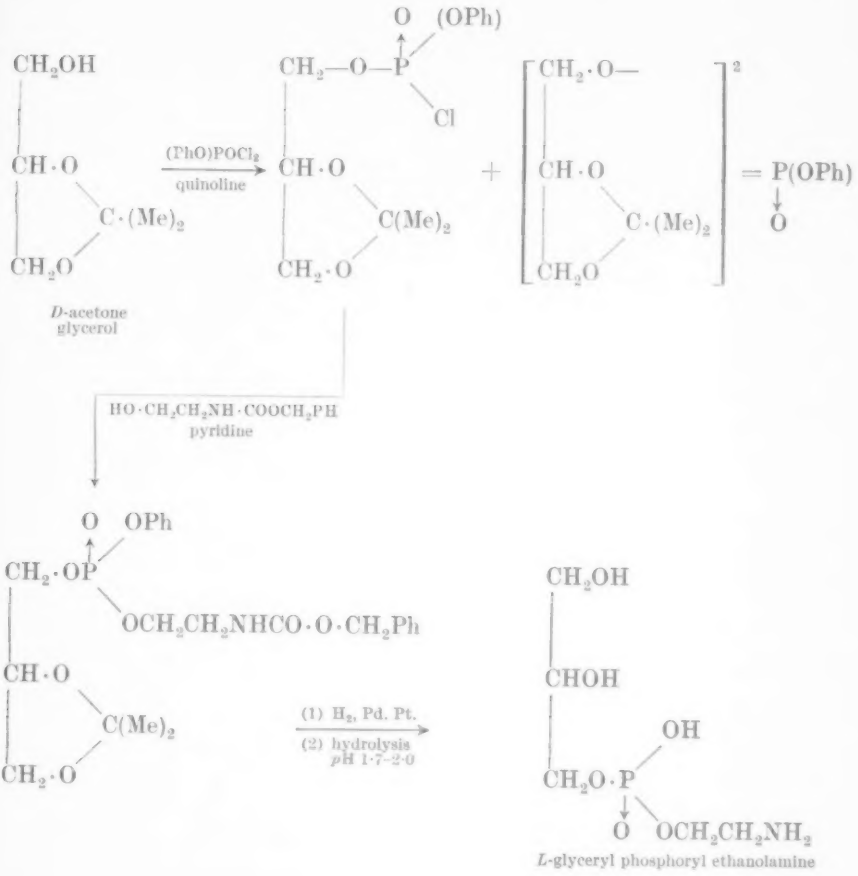
This has been synthesized by Baer and Kates[16] as follows:



The final acetone-phenyl derivative was purified by conversion into the reineckate which, in contrast to other reineckates present, is soluble in ethyl acetate; and after conversion to the sulphate, by means of silver sulphate, the phenyl group was removed by catalytic hydrogenolysis, and the acetone group was finally removed in water at *p*H 1·5. This order of removal of protecting groups is important, in order to avoid acyl migration. The synthetic product was found to be identical with a product obtained from autolysed beef pancreas. It is a viscous liquid, soluble in water, methanol, and ethanol, but insoluble in acetone, ether, or benzene.

L-α-glycerylphosphoryl ethanolamine (*L*-glyceryl-2-aminoethyl phosphate)

This has been synthesized as follows by BAER and STANCER[61] in an overall yield of 33–35 per cent.

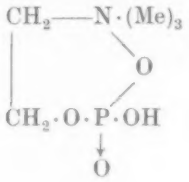


The final crystalline product, M.pt. 86–87°, $[\alpha]_D^{26}$ — 2·9 was identified with that obtained from plasmalogens, thus establishing that the latter belong to the *L* series.

Choline phosphate

Choline phosphate has been prepared by SCHMIDT,[62] PLIMMER and BURCH,[63] RENSHAW and WARE,[64] by the action of phosphorus oxychloride on choline chloride, the phosphate being isolated as the barium salt of phosphorylcholine

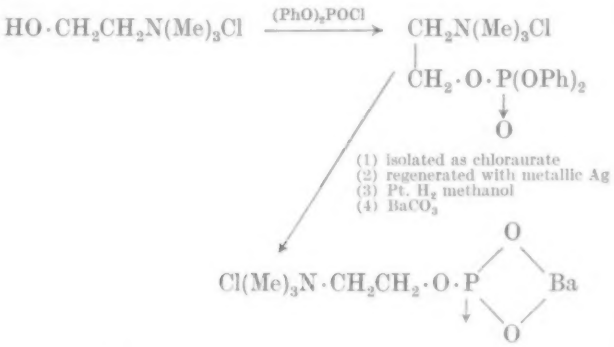
chloride. PLIMMER and BURCH prepared other salts, including the calcium salt from which they prepared free choline phosphate in the anhydro form.



Phosphoric acid and phosphorus pentoxide have also been used to phosphorylate choline chloride by PLIMMER and BURCH (*loc. cit.*), GRÜN and LIMPÄCHER,[65] JACKSON,[66] INUKAI and NAKAHARA,[67] and BEZNAK and CHAIN,[68] and ethyl metaphosphate has been used by ABDERHALDEN, POFFRATH, and BICKEL.[69]

Ethylene chlorohydrin has also been phosphorylated, and the product treated with trimethylamine (PLIMMER and BURCH; JACKSON, ABDERHALDEN, *et al.* (*loc. cit.*), RENSHAW and HOPKINS,[70] LANGHELD[71] and MCMEEKIN.[72]

The above procedures gave poor yields even in the best cases, and BAER and MCARTHUR[73] employed the following improved synthesis:

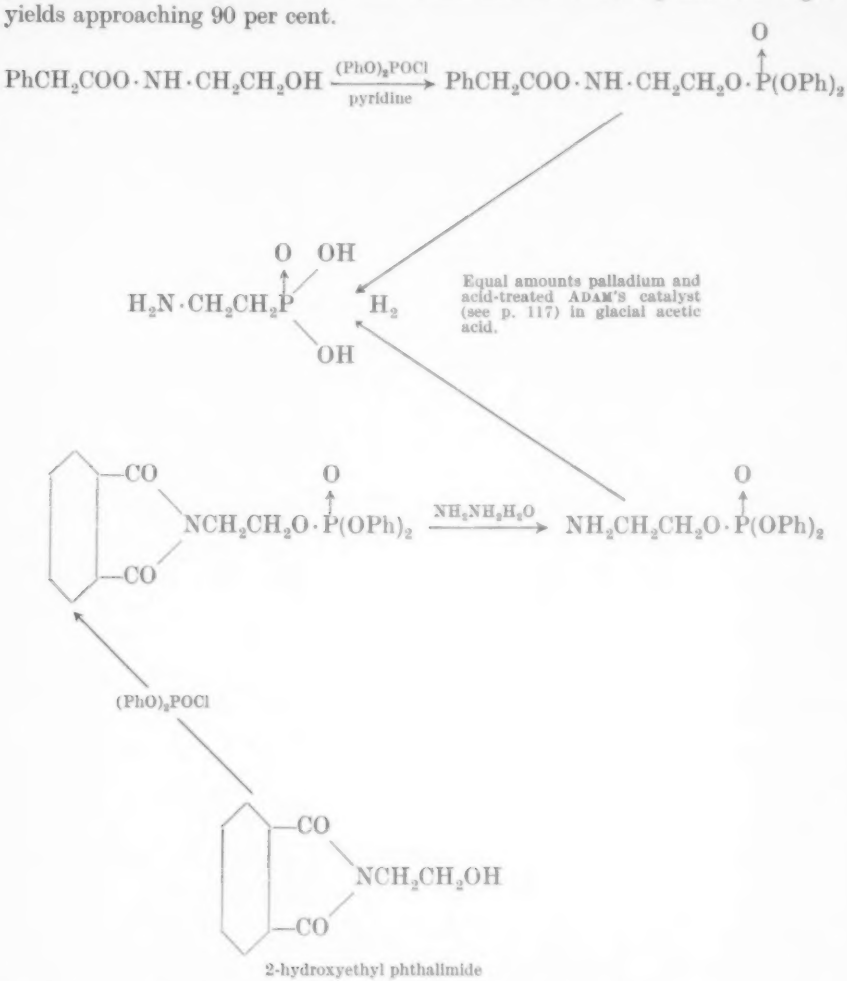


In a later paper, BAER[74] considerably simplified the procedure, and hydrolysed off the protecting phenyl groups by boiling with baryta, thus avoiding the expensive and tedious chloraurate purification, and the catalytic hydrogenolysis.

Ethanolamine phosphate (2-aminoethyl dihydrogen phosphate)

The first synthesis is due to OUTHOUSE,[75] who prepared it by the action of phosphorus oxychloride on ethanolamine, followed by hydrolysis. The phosphate was isolated as the barium salt, from which the free acid was regenerated by treatment with sulphuric acid. PLIMMER and BURCH[63] used much the same method, and also used a mixture of phosphoric acid and phosphorus pentoxide as a phosphorylating agent. CHRISTENSEN[30] obtained the same product by the action of orthophosphoric acid on ethyleneimine, and CHERBULIEZ and WENIGER[47] obtained it by the action of a polyphosphoric acid on ethanolamine. None of the above methods is satisfactory from the point of view of quality or

yield, and BEVAN and MALKIN[76] have used the two following methods to give yields approaching 90 per cent.



2-hydroxyethyl phthalimide

Whilst this account was being prepared, BAER and STANCER[77] published a synthesis which is identical with the first of the above methods, except that the hydrogenolysis is carried out in two stages. The first stage employed palladium in alcohol and the second ADAM'S catalyst in glacial acetic acid. BEVAN and MALKIN noted in their work that when palladium or ADAM'S catalyst and alcohol were used in the hydrogenolysis stage of either of their methods, monophenylethanolamine phosphate separated from solution, and its insolubility in alcohol is undoubtedly the cause of the incomplete hydrogenolysis implied by BAER and STANCER'S two stage method. Hydrogenolysis in alcohol is, in fact, an excellent method for the preparation of the monophenyl derivative.

Ethanolamine phosphate and its monophenyl derivative are both very

soluble in water, slightly soluble in boiling methanol, and practically insoluble in boiling ethanol. They give well defined crystals when ethanol is added dropwise to a concentrated aqueous solution, M.pt. phosphate 243°, phenyl derivative, 253–5°. Diphenyl phosphoryl ethanolamine hydrobromide, M.pt. 115–16° can be prepared from diphenyl phosphoryl carbobenzoxyethanolamine, by treatment with hydrobromic acid in glacial acetic acid.

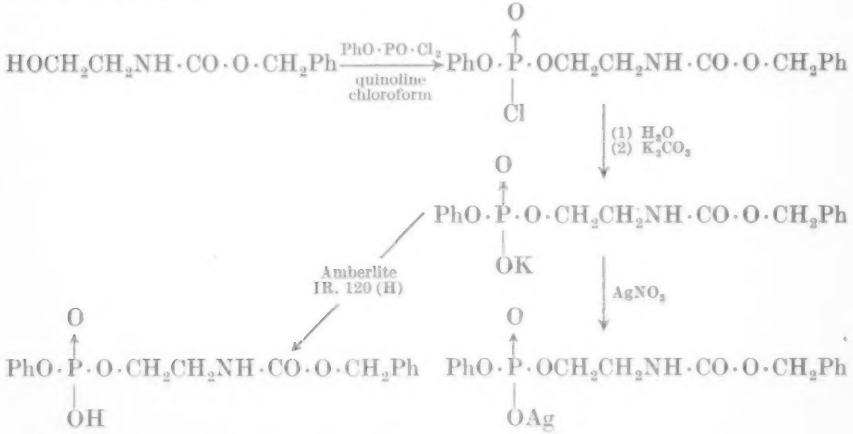
Serine-3-phosphate

Serine phosphate has been obtained by LEVENE and SCHORMÜLLER[78] in low yield, by the action of phosphoric acid and phosphorus pentoxide on serine, and by the action of phosphorus oxychloride on benzylidine serine. PLIMMER[79] using the former method, in an autoclave at 20 lb. pressure at 100°C, obtained yields of 8–21 per cent. BEVAN and MALKIN[76] obtained yields of 85 per cent by treating *DL*-N-carbobenzoxy serine benzyl ester[31] with diphenylphosphoryl chloride in pyridine, and removing the protecting groups by hydrogenolysis with 1/1, palladium/ADAM'S catalyst.

Serine phosphate is soluble in water, from which it can be precipitated by dropwise addition of ethanol. It can be crystallized from aqueous acetic acid by concentration under reduced pressure below 40°C; M.pt. 164–5°.

Silver salt of phenyl phosphoryl-N-carbobenzoxy ethanolamine

This was prepared by BAYLIS, BEVAN, and MALKIN[23] as follows:

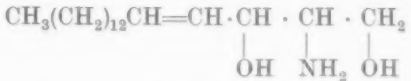


The free acid crystallizes from ether, M.pt. 75–76°.

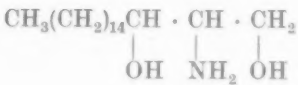
VIII. SPHINGOLIPIDS

No synthetic work has been reported on sphingolipids themselves, but several groups of workers have synthesized dihydrosphingosine, and two syntheses of sphingosine have been announced. On the basis of the behaviour of dihydrosphingosine and its N-acyl derivatives towards periodic acid, CARTER, GLICK,

NORRIS, and PHILLIPS[80] had established the following structures for these two substances:



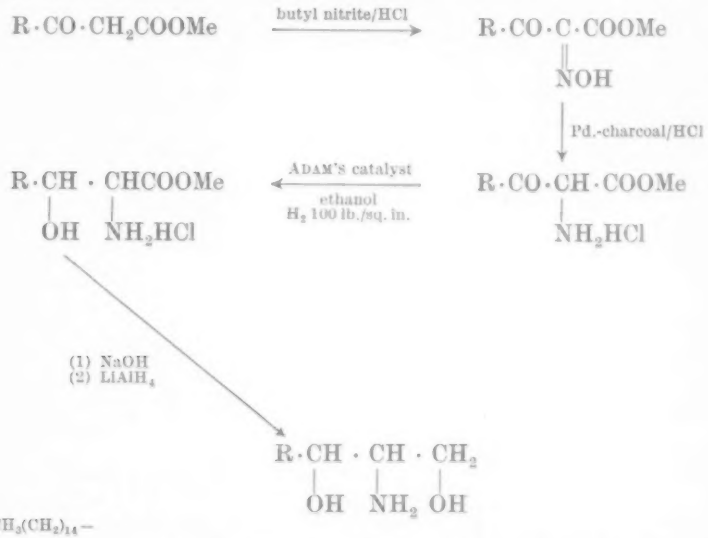
Sphingosine



Dihydrosphingosine

Dihydrosphingosine

The first published synthesis was that of GREGORY and MALKIN,[81] who oximinated methyl 3-keto-octadecanoate[82] and reduced the oxime stepwise as indicated below:

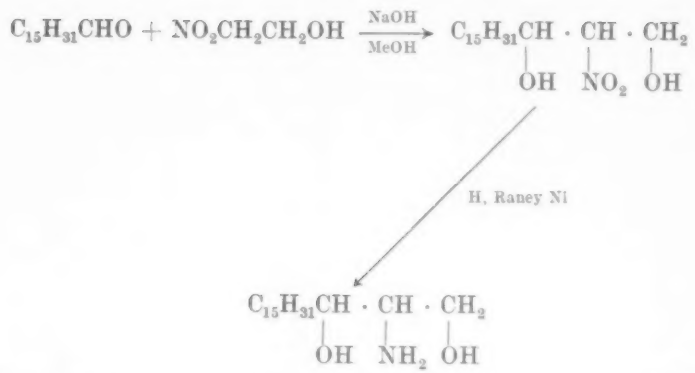


R = $CH_3(CH_2)_{14}$–

The N-acetyl, triacetyl, and the tribenzoyl derivatives had M.pts. remarkably close to those of the naturally occurring dihydrosphingosine prepared by CARTER *et al.*,[80] despite the fact that the synthetic material is probably a mixture of the two possible racemates. It has been suggested, however, by FODOR, BRUCKNER, KISS, and OHEGYI[83] that the orientation of the keto and oxime groups predetermines the position of the hydroxy and amino groups in the reduction product, leading to the formation of one racemate only. The M.pts. of the above derivatives indicate that the synthetic product has mainly the erythro configuration, which is now known to be that of the natural dihydrosphingosine.[84]

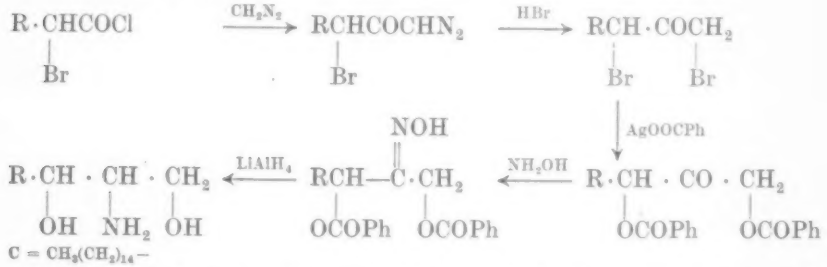
A little later, FISHER[85] was able to reduce the above oxime, directly to dihydrosphingosine by means of lithium aluminium hydride.

GROB, JENNY, and UTZINGER[86] had meantime carried out the following synthesis.



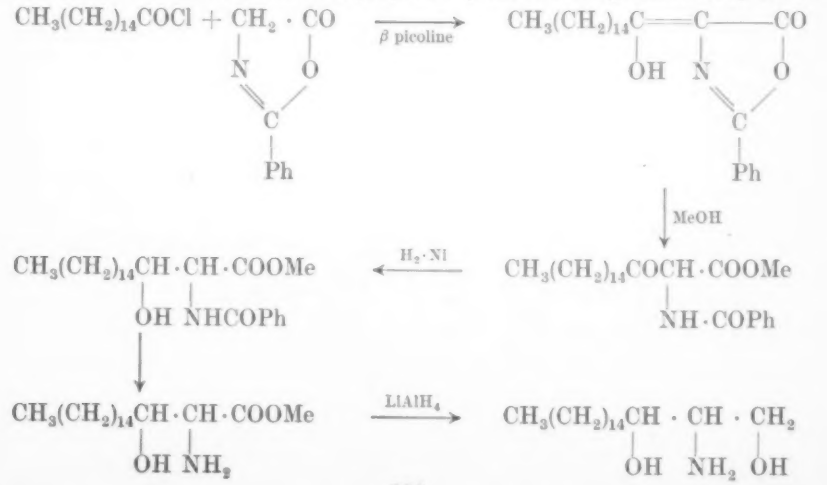
and EGERTON, GREGORY, and MALKIN,[87] who had independently studied this method, and who used sodium methylate in the first stage and ADAM's catalyst in the second, considerably improved the yield in all stages, including the preparation of the aldehyde.

PROSTENIK and STANECER[88] accomplished a synthesis as follows:



Yields were not good, but a number of pure derivatives were made and the authors concluded that only one racemate was present.

CARTER, HARRISON, and SHAPIRO[89] prepared the erythro and threo forms of 2-amino-1:3-dihydroxy octadecane via the erythro and threo forms of α-amino β-hydroxy stearic acid, which were prepared by the following azlactone synthesis.



The racemic α-amino β-hydroxy stearic acids were characterized as the threo and erythro isomers by comparison of the properties of a number of derivatives with the corresponding derivatives of threonine and allothreonine (erythro). The assignment of configuration was based on characteristic differences in the infra-red and in the M.pt. and solubility behaviour of the two isomers, which were exactly duplicated in the C_{18} series. They, moreover, observed that the erythro-2-amino-1:3-dihydroxy octadecane and also natural dihydrosphingosine, readily yielded a tribenzoyl derivative, whereas the threo compound yielded only a dibenzoyl derivative, and they concluded that natural dihydrosphingosine belonged to the erythro series.

JENNY and GROB,[90] a little later, indicated the scheme shown on p. 132 for the preparation of both threo and erythro forms, and prepared the threo isomer by this method.

The unwanted isomers (*b*) and (*d*) can be destroyed by the action of periodic acid on the N-acetyl derivatives, which leaves (*a*) and (*c*) unattacked.

The stereochemical course of the epoxide ring opening by ammonia had been previously studied by DICKEY, FICKETT, and LUCAS,[91] who found a reversal of configuration at the carbon atom attacked by ammonia.

In a later paper, JENNY and GROB[92] carried out the synthesis of the erythro isomer, by a slight modification of the above method, starting from trans-2-octadecenoic acid.*

$$CH_3(CH_2)_{14}CH{=}CH\cdot COOH \xrightarrow{SOCl_2} CH_3(CH_2)_{14}CH{=}CH\cdot COCl$$

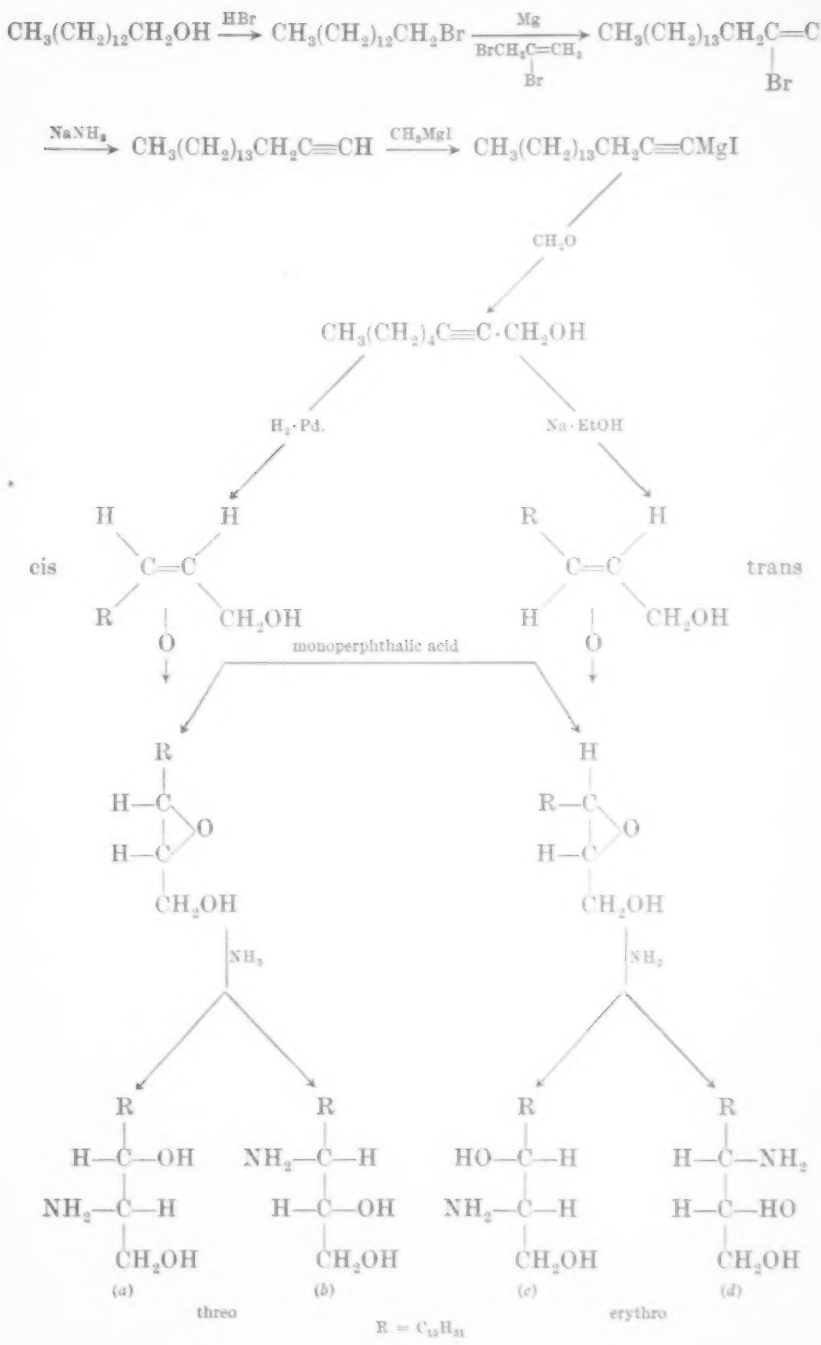
$$\xrightarrow{LiAlH_4} CH_3(CH_2)_{14}CH{=}CH\cdot CH_2OH$$

via epoxide and ring opening with NH_3 as shown on p. 132

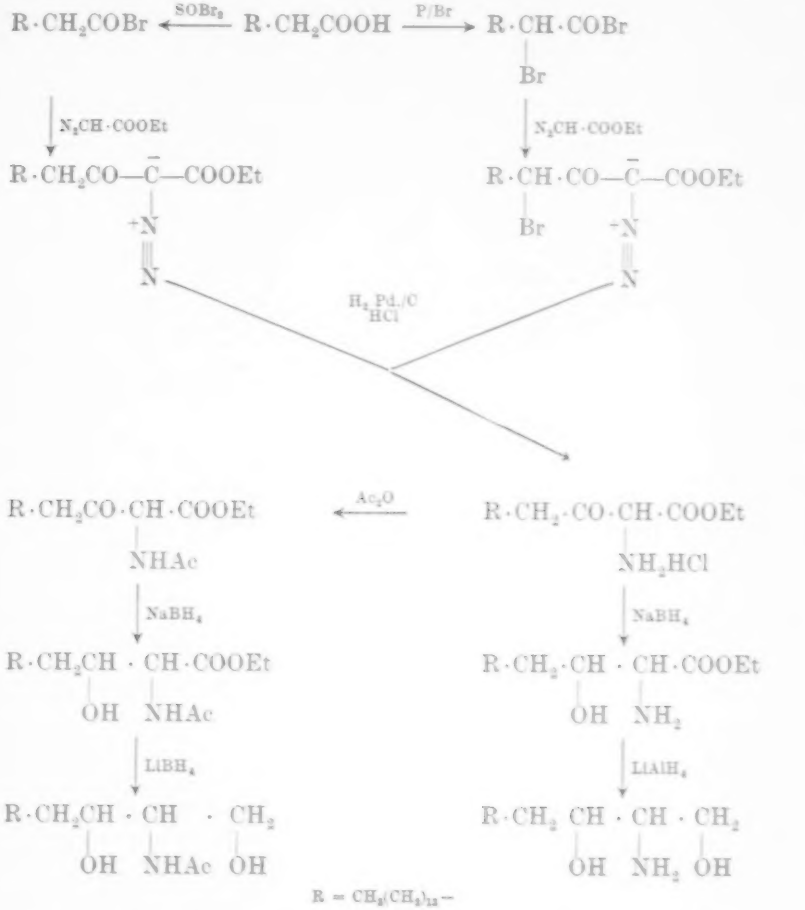
$$CH_3(CH_2)_{14}\underset{OH}{CH}\cdot\underset{NH_2}{CH}\cdot\underset{OH}{CH_2}$$

erythro

* GROB and JENNY omit to mention that these results fully confirm the earlier work of EGERTON *et al.*[87] which they had previously criticized.[94]

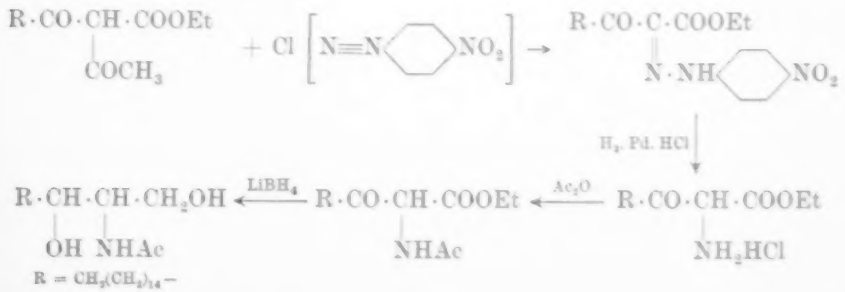

CH3(CH2)12CH2OH
HBr
CH3(CH2)12CH2Br
Mg
BrCH2C=CH2
Br
CH3(CH2)13CH2C=CH2
Br
NaNH2
CH3(CH2)13CH2C≡CH
CH3MgI
CH3(CH2)13CH2C≡CMgI
CH2O
CH3(CH2)4C≡C·CH2OH
H2·Pd.
Na·EtOH
cis
trans
monoperphthalic acid
NH3
NH3
threo
erythro
R = C13H31

A further synthesis of *DL*-erythrodihydrosphingosine was achieved by SALLAY, DUTKA, and FODOR[93] by the following route:

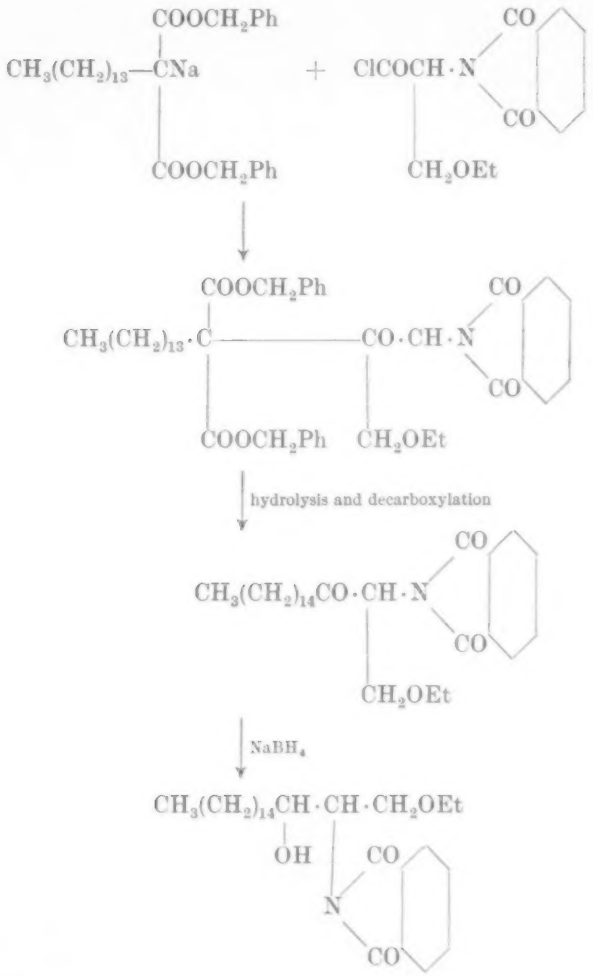


The erythro form was separated from the end product by crystallization.

SALLAY and DUTKA[95] have also utilized the Japp-Klingermann reaction in the following synthesis:



and finally PROSTENIK and STANACEV[96] have described a synthesis from a derivative of *DL* serine as follows:

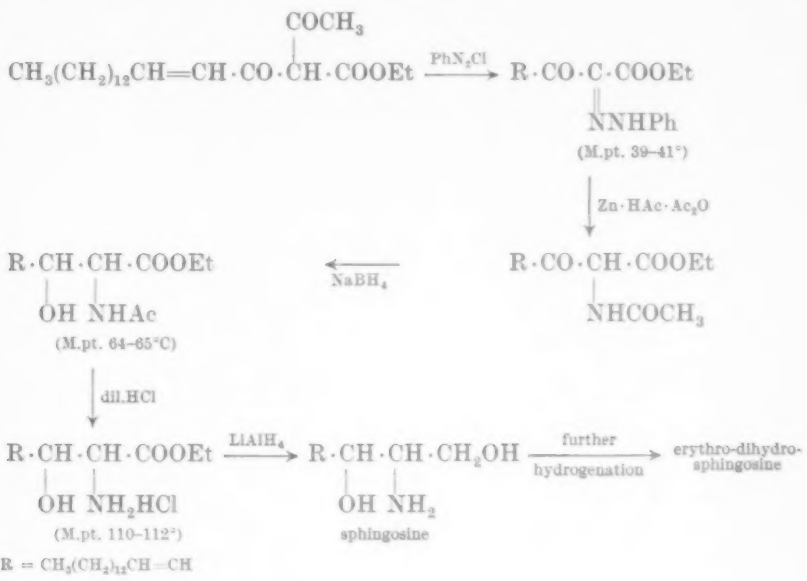


yielding the N-phthalyl-1-0-ethyl derivative of dihydrosphingosine.

Sphingosine

GREGORY and MALKIN[81] obtained racemic 2-amino-octadec-4-ene 1:3-diols by condensation of nitroethanol with hexadec-2-ene-1-al, followed by reduction with lithium aluminium hydride, but the yield was low, and subsequent work indicates that this is not likely to prove a practicable synthesis.

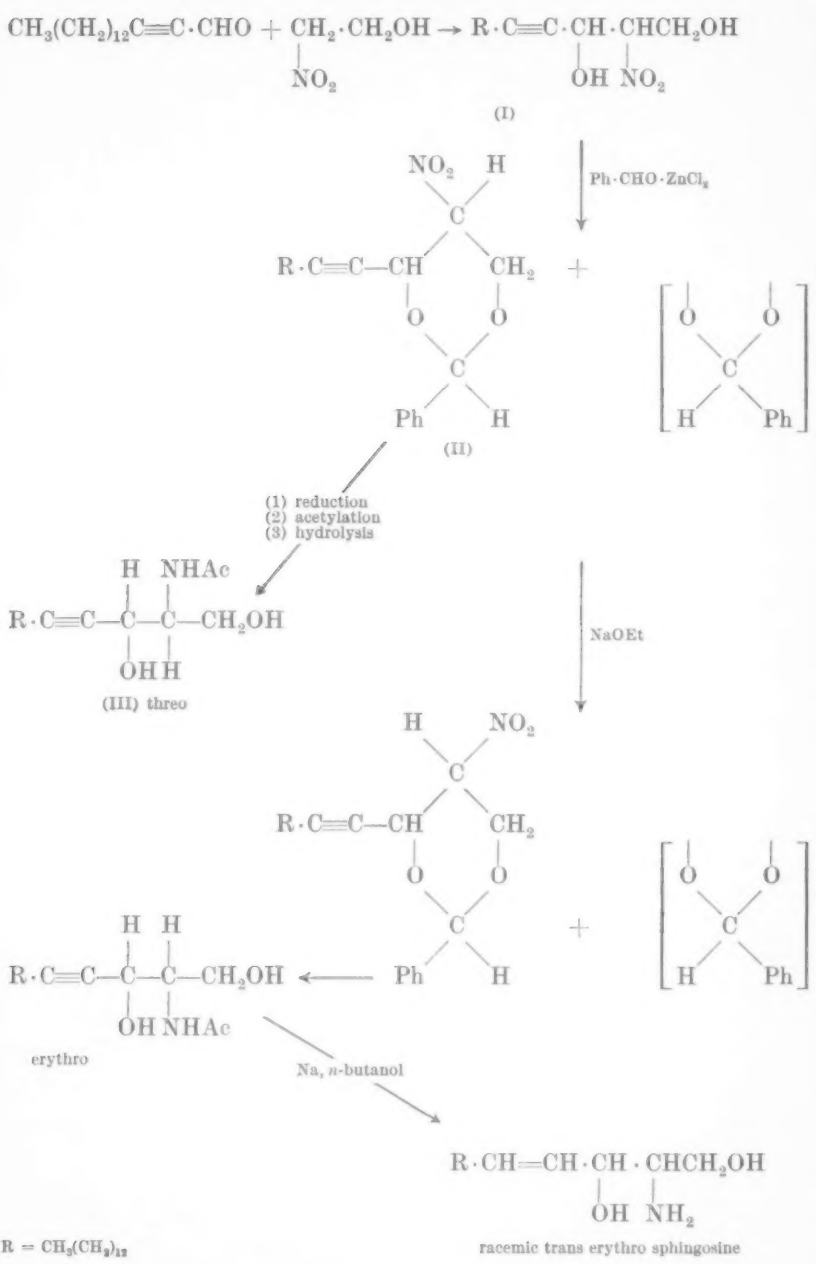
In 1954, SHAPIRO and SEGAL[97] reported a synthesis by the Japp-Klingerman reaction as follows; but full details are not yet available.



Quite recently, GROB and GADIENT[98] have carried out the following synthesis: 2-Hexadecyne-1-al was base catalytically condensed with nitroethanol to yield 1:3-dihydroxy-2-nitro-4-octadecyne, I, which when condensed with benzaldehyde yielded two isomeric benzylidine compounds, II, both of which were converted into threo 1:3-dihydroxy-2-acetylamino-4-octadecyne, III, on reduction of the nitro group, followed by acetylation and hydrolysis of the cyclic acetal. The configuration follows from the conversion of III into threo N-acetyl dihydrosphingosine on hydrogenation (Pd).

Partial reduction of the triple bond in III, by sodium in *n*-butanol, yielded racemic threo sphingosine, which was characterized as the triacetyl compound.

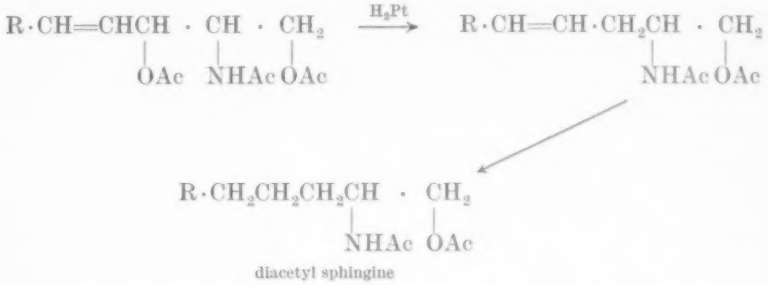
The above threo benzylidine compounds were converted by alcoholic sodium ethoxide into the isomeric erythro isomers, from which racemic erythro sphingosine was obtained by an identical series of reactions.



KISS, FODOR, and BANFI,[99] SALLAY,[100] FISHER,[85] and AHMAD[101] are actively engaged on this synthesis.

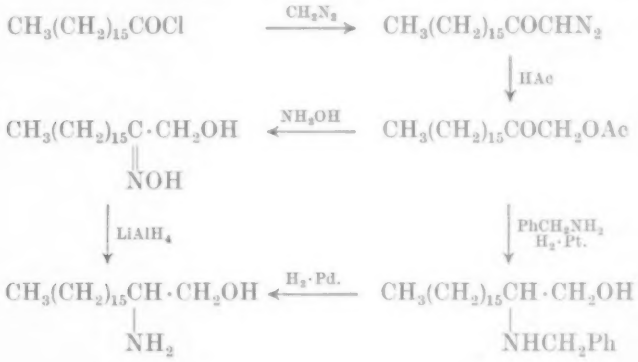
Sphingine

CARTER and HUMISTON[102] showed that when triacetyl sphingosine was catalytically reduced, a certain amount of hydrogenolysis of the allylic acetoxyl group takes place, in addition to the normal reduction to triacetyl dihydrosphingosine, yielding diacetyl sphingine.



diacetyl sphingine

DL sphingine has previously been synthesized by CARTER, NORRIS, and ROCKWELL[103] by the catalytic reduction of methyl α-aminostearate using Raney nickel and hydrogen under high pressure, and a further synthesis has been announced by SUNKO and PROSTENIK[104] as follows:



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6

THE LECITHINASES

Donald J. Hanahan

CONTENTS

		Page
I.	INTRODUCTION	142
	A. Nomenclature	142
II.	HYDROLYTIC ENZYMES	144
	A. Lecithinase *A*	144
	1. General characteristics and typical reaction systems	144
	2. Substrate specificity and site of action	147
	3. Application to biochemical problems	149
	(*a*) Attempted synthesis of specifically labelled lecithins	149
	(*b*) Positional and metabolic asymmetry of lecithins	149
	4. Purification and properties	151
	B. Lysolecithinase *B*	152
	1. General remarks on reaction	153
	2. Reaction systems and substrate specificity	153
	3. Isolation of enzymes	157
	C. Lecithinase *C*	158
	1. Primary mode of attack	159
	2. Activation of enzyme and substrate requirements	162
	D. Lecithinase *D*	162
	1. General considerations and specificity of action	163
	2. Purification procedures	165
III.	OTHER POSSIBLE ENZYMES INVOLVED IN LECITHIN METABOLISM	165
	A. Hydrolytic type	166
	B. Isomerase	167
	C. Synthetic enzymes	169
	1. Phosphate and glycerophosphate to lecithin	170
	2. Phosphorylcholine conversion	170
	D. Oxidation-reduction enzymes	171
	1. Phospholipide involvement in mitochondrial reactions	172
	2. Evidence for lecithin dehydrogenases	173
IV.	SUMMARY	174
	REFERENCES	175

6

THE LECITHINASES

Donald J. Hanahan

I. Introduction

Lecithin, a diacyl derivative of *L*-α-glyceryphosphorylcholine (Fig. 1) is one of the more widely distributed of the complex lipides and due to its relative ease of preparation and identification has enjoyed the most widespread attention in studies on fat metabolism. Although its specific role, if any, in lipide metabolism has not been defined, there is sufficient evidence to implicate it in some manner in the lipide turnover picture in mammalian tissues. As a natural adjunct to such studies, the behaviour of enzymes which can attack these complex lipides has interested many investigators. In a classical sense, the lecithinases are defined as those enzymes which can catalyse the hydrolysis of each of the four ester bonds of the model compound, lecithin. When evaluated on a more modern basis, it is clearly evident that one must consider other enzymes which can affect this molecule, i.e. enzymes involved in the synthesis of lecithin, transferases, dehydrogenases, etc. Although the main emphasis here will be directed towards the hydrolytic type lecithinases, the evidence for the existence of other enzymes will be presented.

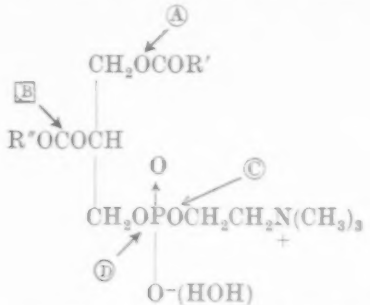
In a discussion of the hydrolytic-type lecithinases, it is well to emphasize that much of the early literature on this subject, must be viewed with scepticism as little supporting chemical evidence was supplied. Only in the light of more recent evidence can the older literature be carefully assayed. A particular case in point is that of lecithinase *A*, which has long been thought capable of removing unsaturated fatty acids from lecithin, but only recently was its exact mode of action explained. This difficulty is attributable to early reports that an oil was released from lecithin by action of lecithinase *A*, and this was interpreted repeatedly as supporting evidence for unsaturation and for fatty-acid release. In addition, the use of ill-defined assay systems, such as the haemolysin test or toxicity levels in animals, have not aided in the elucidation of the nature of the reaction. Hence, in this review an attempt will be made to present and correlate the more definitive information on the mode and specificity of action of these enzymes.

A. Nomenclature

The terminology for the hydrolytic enzymes in which the designation for the site of attack was indicated by the letters *A*, *B*, *C*, and *D*, was proposed in 1933 by Contardi and Ercoli.[14] At that time, evidence for the existence of only

two of these enzymes, *A* and *B*, was indicated. A modification of this scheme to fit more recent findings will be used here.

In considering the most suitable nomenclature system for these enzymes, one is hampered by the lack of general suitability of any particular system. Until such time as more detailed information is available and for the reason cited below, the following scheme, which is in part a modification of that of CONTARDI and ERCOLI and the suggestions of CELMER and CARTER,[9] will be used:



R', R" = unsaturated and/or saturated long chain fatty acids.

Fig. 1. Site of action of lecithinases *A*, *C*, and *D*. *B* now considered a lysolecithinase.

The major change in this enzyme nomenclature is the designation of only three established lecithinases, namely, *A*, *C*, and *D*. Inasmuch as sufficient evidence is now available, the term lecithinase *B* should be abandoned and lysolecithinase *B* should be substituted. Also, the variations among investigators as to the correct designation for the terms lecithinase *C* and *D* should be resolved. With all due respect to priority, it still seems preferable to associate lecithinase *C* with the choline-removing enzyme and lecithinase *D* with the diglyceride-cleaving enzyme. Hence, in this article the following actions are attributed to the letter designations in the above formulation: *A*, release of one mole of fatty acid from the lecithin molecule; *B*, release of one mole of fatty acid from the lysolecithin molecule; *C*, removal of the nitrogen base from the lecithin molecule; *D*, release of the phosphorylated base (or formation of diglyceride) from lecithin.

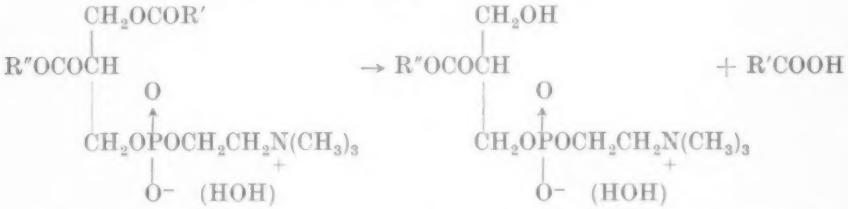
Although this system of nomenclature is certainly not specific in all respects and will have its inconsistencies, it has many points in its favour. Foremost among these is the fact that, to date, the purest substrates most easily available to all workers have been the lecithins which can be prepared in a reasonable state of purity in most laboratories. Consequently, they serve as a rather standardized substrate. Other phospholipides with different functional groups for use in test systems have not been available either in sufficient quantity or in a desirable state of purity and thus this fact has imposed a limitation. Furthermore, this system of nomenclature has been in use for a number of years and among workers in the field there appears to be a reasonable understanding as to the meaning of the sites of action. Finally, although evidence is

becoming available on the mode and specificity of action of these enzymes, their purification has not progressed to a level commensurate with a detailed revision of the system of nomenclature, such as the use of the term phospholipase.[22]

II. Hydrolytic Enzymes

A. Lecithinase A

This enzyme specifically effects the hydrolysis of the ester bond at the α′ position of lecithin with the concomitant release of free fatty acid and formation of lysolecithin. Both unsaturated and saturated acids can be removed.



Among the various lecithinases, lecithinase *A* has enjoyed the most widespread attention in investigation. It has been detected in pancreas, snake venoms, bee and wasp venoms, kidney and other tissues.[14] Recent evidence shows that it is present in bacteria[34] but no reports on its existence in plants have been forthcoming.

Although it has been repeatedly stated in the literature that only unsaturated fatty acids were released, it was not until the work of Fairbairn[22] that any definite evidence was presented as to the chemical nature of the released fatty acid. More recent evidence[31] has shown that it is not primarily the nature of the fatty acid that influences the action of lecithinase *A* but rather the position, namely whether α′ or β.

1. *General characteristics and typical reaction systems*

Prior to the introduction by Fairbairn[22] of a titrimetric scheme to follow the reaction, most investigators had used haemolysis of red blood cells by "lysolecithin" as the assay system. This method at best aided little in the interpretation of the enzymatic reaction other than stating that the reaction mixture possessed haemolytic activity. In many instances, the presence of haemolytic substances other than lysolecithin in the crude tissue extracts used as enzyme sources could not be excluded.

In 1945 Fairbairn[22] reported the results of his studies on the lecithinase in the venom of the cottonmouth moccasin snake (*Agkistrodon piscivorus* L.). In his test system, the phospholipide and enzyme were mixed in an aqueous buffer at *p*H 7·0 and incubated at 37°. At the end of the incubation period the free fatty acids were isolated and titrated with dilute alkali. Specifically the test system consisted of an aqueous emulsion of a crude phospholipide fraction from brain (3·0 per cent P, choline/P, 0·27, 17–20 per cent acetal phospholipides and

3·6 per cent cholesterol) to which the venom in an aqueous buffer was added and the mixture incubated at 37°. After the desired intervals, alcohol or a lead salt was added to stop the reaction and the fatty acid isolated by an involved scheme which required complete removal of the buffer and any remaining phospholipide. No quantitative separation of the lysolecithins could be achieved, but an isolated sample appeared to be a typical lysolecithin. No supporting evidence was given as to the structure of the lysolecithin. It is interesting to note that FAIRBAIRN found only unsaturated fatty acids in the free fatty-acid

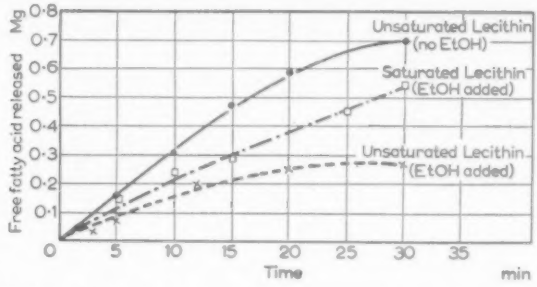


Fig. 2. Action of *Naia naia* venom on unsaturated and saturated lecithin in 95 per cent diethyl ether-5 per cent alcohol. The control, containing unsaturated lecithin plus venom, was run in ether only.
Taken from HANAHAN *et al. J. Biol. Chem.* **206**, 431 (1954).

fraction, while the lysolecithin fraction had a negligible iodine-absorption value. This supports the concept of positional asymmetry of fatty acids on lecithins noted in other tissues (*cf.* Section III*A* below).

In the course of an investigation on the mode of action of lecithinase *A* of pancreas on highly purified egg lecithin, HANAHAN[76] observed that the reaction would proceed in a clear, moist diethyl-ether solution and obeyed the normal criteria for an enzyme-catalysed system. This technique provided good evidence for the existence of an enzyme-substrate complex. This was proved by the following observations: (*a*) the enzyme itself was not soluble in diethyl ether alone; (*b*) a mixture of the substrate in diethyl ether with the enzyme in an aqueous phase caused the initiation, upon complete mixing, of an enzymatic degradation in the separated ether phase. This system allows a convenient means for the studies of the kinetics of this reaction and a facile route to the separation of the products, as the lysolecithin is ether insoluble, while the free fatty acids are ether soluble.

Utilizing a direct titrimetric procedure for following the progress of the reaction, HANAHAN *et al.*[30] made a detailed examination of the products of the action of *Crotalus adamanteus* venom and *Naia naia* venom on (dipalmitoleyl)-L-α-lecithin (yeast lecithin). In this reaction system, it is interesting to note that a precipitate forms in the reaction near 40 per cent completion, but apparently does not affect the course of the reaction (Figs. 2 and 3). This precipitate is composed of a mixture of the lysolecithin and unreacted substrate, which ultimately is completely converted to lysolecithin. A study of this

precipitate formation showed that the enzyme was wholly associated with this fraction and evidently was able to continue to act in such a medium (Fig. 4). In following the action of lecithinase on saturated lecithins, which are insoluble

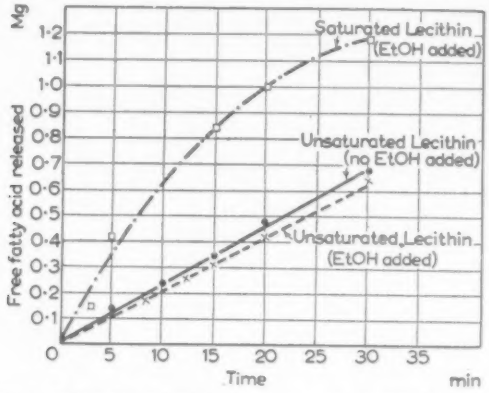


Fig. 3. Action of *Crotalus adamanteus* venom on unsaturated and saturated lecithin in 95 per cent diethyl ether-5 per cent alcohol. The control, containing unsaturated lecithin plus venom, was run in ether only.
Taken from HANAHAN *et al. J. Biol. Chem.* **206**, 431 (1954).

in diethyl ether alone but soluble 94 per cent Et_2O—5 per cent EtOH—1 per cent H_2O mixture, it was observed that the enzyme was fully active.

In general, this ether system represents a very convenient and rapid route to the large scale preparation of lysolecithins. For example, 5 gm of egg or

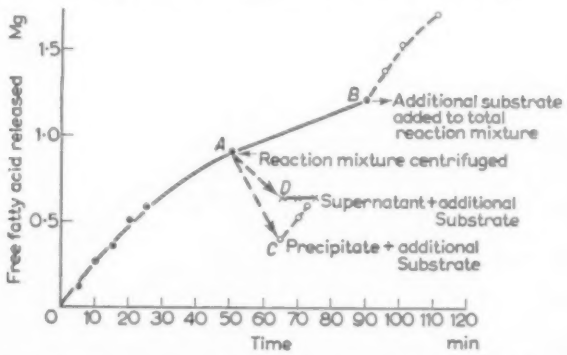


Fig. 4. Location of enzyme in reaction mixture. At Point *A* in a reaction of *Naia naia* venom on unsaturated lecithin in diethyl ether, a precipitate had formed. Point *B* represents another similar reaction, which was allowed to go to essential completion and additional substrate was added.
Taken from HANAHAN *et al. J. Biol. Chem.* **206**, 413 (1954).

yeast lecithin dissolved in 250 ml diethyl ether may be completely degraded within 2 hr by the addition of 20 mg of *Crotalus adamenteus* venom dissolved in 0·5 ml water. The resulting precipitate from this enzymatic reaction is washed several times with diethyl ether and then may be crystallized to a pure state from a chloroform or absolute alcohol solution. The lecithinase *A* from

snake venom, pancreatin, and fresh pancreas have been found to be active in this system. A lecithinase *A* present in a bacterial system is reported not to be active in an ether solution.[34]

In a study of the reactivity of a lecithinase *A* present in *Serratia plymuthicum* upon egg lecithin, HAYAISHI and KORNBERG[34] followed the action through a series of reactions wherein (*a*) the lysolecithins first formed were degraded by a potent lysolecithinase to glycerylphosphorylcholine (GPC) and free fatty acid and (*b*) the GPC was further decomposed by a purified GPC diesterase to glycerophosphate and free choline. The assay for free choline was used as an index of the activity of the lecithinase *A*. Although this system indicates the versatility of the enzyme employed, the number of enzymes required for an assay would tend to limit the general usefulness of this technique. In their system, HAYAISHI and KORNBERG found a requirement for calcium ions. This is of interest inasmuch as such a requirement is not noted in the ether system. One possible explanation is that in an ether solution this solvent must effectively remove free fatty acids from the vicinity of the enzyme surface, whereas in an aqueous medium the calcium ions perform a similar function. This same requirement for calcium ion had been noted by DESNUELLE and co-workers[18] in their studies on the action of a lipase on triglycerides in an aqueous medium. They concluded that there was a requirement for calcium ion due to the fact that the free fatty acid formed must be swept away from the enzyme surface or there would be an inhibition of the reaction. Of interest is the observation by DESNUELLE[19] that pancreatic lipase is not active in or activated by diethyl ether.

2. *Substrate specificity and site of action*

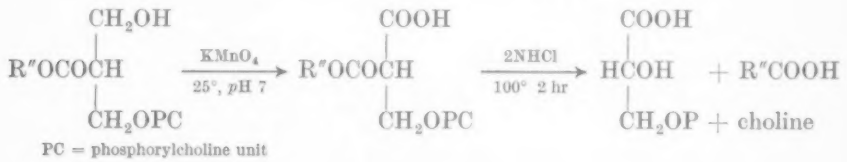
Substrates. This enzyme system is capable of attacking both highly purified saturated and unsaturated lecithins.[30] It apparently exerts little or no activity on cerebrosides, sphingomyelins, acetal phospholipides, or lysolecithins.[22] As regards its activity towards cephalins, in particular phosphatidyl ethanolamine, the evidence is rather equivocal. LEVENE and ROLF[48] had shown that lecithinase *A* can attack the egg yolk phospholipides with the production of lysolecithin and lysocephalin, while CHARGAFF and COHEN[10] presented evidence for its inactivity towards purified cephalins. Utilizing crude brain phospholipides which contained ethanolamine phospholipides, etc., FAIRBAIRN[22] concluded that cephalins as well as lecithins were attacked. Recent evidence by LONG and PENNY[55] indicates that lecithinase *A* may be able to attack a purified phosphatidyl ethanolamine in an ammoniacal ether solution though the reaction is much slower than for the lecithin substrate. It will be interesting to learn more of this reaction system and if the specificity of the lecithinase *A* is the same on the phosphatidyl ethanolamine as on the lecithin molecule.

(*b*) *Site of action.* Until recently,[31] the site of action of lecithinase *A* on lecithin had never been proved. With the availability of pure, homogeneous lysolecithins, produced enzymatically by the action of snake venoms on pure lecithin it was possible to make a detailed study of the position of the free alcohol group.

LEVENE and MEHLTRETTER[50] reported unsuccessful attempts to prepare a derivative of lysolecithin formed by the action of snake venom on egg yolk lecithin. The only derivative on which these same authors reported success was the preparation of lecithin from lysolecithin by the reaction of this compound under anhydrous conditions with fatty acid anhydrides and sodium acetate. However, numerous attempts to repeat this synthesis in our own laboratory have failed.

In a detailed study,[31] the use of other reagents, i.e., *p*-nitrobenzoylchloride, phthalic anhydride, and dihydropyran, for derivative preparation, proved unsuccessful. Another possible approach was to remove the phosphoryl choline from lysolecithin by the use of lecithinase *D*, with the resultant formation of an easily identifiable monoglyceride. However, in confirmation of the indirect evidence obtained by ZAMECNIK *et al.*,[75] on the inactivity of lecithinase *D* toward lysolecithin, no action of this enzyme on pure lysolecithin could be elicited.

The approach which was finally selected as the most feasible and proved successful, involved the use of potassium permanganate to oxidize the free alcohol group to a carboxyl group. After the oxidation at room temperature or 37°, the reaction mixture was subjected to acid hydrolysis for 2 hr and the products isolated and identified. When the lysolecithin resulting from enzymatic action on *L*-α-lecithin was employed, the only products were phosphoglyceric acid, free fatty acid, and free choline. The phosphoglyceric acid was isolated as the barium salt and identified by paper chromatography and elementary analyses. The yield of fatty acid and choline was greater than 90 per cent of theory, while phosphoglyceric acid was isolated in 65–85 per cent yields. These results were interpreted as evidence for the remaining fatty acid on the lysolecithin to be at the β position, while the alcohol group at the α′ (or *C*-1) position was essentially free. The oxidation scheme thus proceeded as follows:



PC = phosphorylcholine unit

If the alcohol group had been on the β position instead, the phosphate ester resulting from this oxidation and hydrolysis would have been dihydroxyacetone phosphate which under the hydrolytic conditions used would have decomposed to methyl glyoxal and inorganic phosphate. In confirmation of these results, LONG and MAGUIRE[53] reported in a preliminary note that oxidation of saturated lysolecithin with dichromate in glacial acetic acid gave essentially the same results. DAVIDSON *et al.*[16] recently confirmed these observations on the oxidative degradation of lysolecithin by $KMnO_4$ and isolated the potassium salt of the resultant acid. They further observed that synthetic dimyristoyl-*L*-α-lecithin could be attacked by lecithinase *A*, but only the *L* form of a

DL-α-lecithin was hydrolysed. Synthetic β-lecithins and β-cephalins were not degraded.

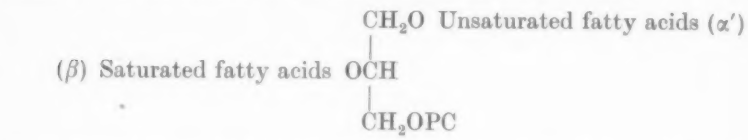
3. *Applications to biochemical problems*

As has been mentioned previously, the specificity of these enzymes should be useful in several ways, among which can be their use in the preparation of certain derivatives, structure proof and studies on intermediate metabolism of the complex lipides.

(*a*) *Attempted synthesis of specifically labelled lecithins.* Through the availability of highly purified lecithins now, it is relatively easy to prepare both unsaturated and saturated lysolecithins[30]. Some examples are β-monopalmitoleyl, β-monopalmityl, and β-monostearoyl-*L*-α-glycerylphosphorylcholine, which can be prepared in 90–95 per cent yields from (dipalmitoleyl)-(dipalmityl)- and (distearoyl)-*L*-α-lecithin by the action of snake venoms in an ethereal medium. Thus, it was hoped through these intermediates to be able to synthesize lecithin with any desired acyl group in the α′ position for use in metabolic studies or enzyme specificity reactions. However, with saturated lysolecithins as the starting material, the use of the following procedures proved unsuccessful: (*a*) LEVENE and MEHLTRETTER'S anhydride synthesis;[50] (*b*) fatty acid anhydrides plus trifluoroacetic acid; (*c*) free fatty acids plus trifluoroacetic acid anhydride; (*d*) replacement of the hydroxyl by halogen through use of oxalyl chloride, triphenylphosphite methiodide or triphenylphosphite plus methyl iodide, coupled with reaction with the silver salt of long chain fatty acid. It is interesting to note that BAER and KATES'S[5] initial attempts at lecithin synthesis involved direct acylation of glycerylphosphorylcholine under a variety of conditions. However, they report only the formation of lysolecithin-like compounds.

(*b*) *Positional and metabolic asymmetry of lecithins.* The possibility that naturally occurring lecithins may contain one molecule each of saturated and unsaturated fatty acids has been of considerable interest for some time. The data of LEVENE and ROLF[48] on the brominated lecithins from liver could be interpreted as indirect support for this theory. However, evidence presented by other investigators casts doubt upon the validity of this proposition.[65, 67] Thus, with the proof that lecithinase *A* attacks only the α′ position of lecithin, a more exact route was then available for a study of this possible arrangement of the fatty acids. Hence, the free acid released in the reaction of lecithinase *A* on any particular purified lecithin would be representative of the fatty acid originally present at the α′ position in intact lecithin. On the same basis the fatty acid of the lysolecithin would be indicative of the acyl group present on the β-ester position in the original lecithin. This technique was applied with success to the nature of the fatty acids on lecithins in beef, rabbit, rat, guinea pig, and dog liver.[29] The data shown in Table 1 indicate that in these sources all the unsaturation was present in the α′ position and all the saturated acids were present in the β position. It is possible that one may find this arrangement does not hold under all conditions, but the evidence at hand indicates that it

represents to a large degree a typical structure for the lecithins in these tissues. Hence, one can formulate the lecithin structure as follows:



Thus there appears to be a position asymmetry to the fatty acids on the lecithin of liver and of egg.

On the basis of this evidence it was of considerable interest to investigate the possible asymmetric *in vivo* incorporation of a labelled saturated fatty

Table 1. Composition of lysolecithins and free fatty acids formed by lecithinase A action on purified liver lecithins

	Beef	*Rabbit*	*Rat*	*Guinea pig*	*Dog*
Lysolecithins*					
P, %	5·61	5·74	5·64	5·04	4·80
N, %	2·54	2·64	2·52	2·25	2·18
Choline, %	21·2	22·1	21·5	19·0	18·7
N:P, *molar ratio*	1·0	1·01	1·00	0·98	1·00
Choline-P, *molar ratio*	0·98	1·00	0·98	0·98	1·00
Free fatty acids					
Neutral equivalent	296	284	310	254	320
Hydrogen No., calculated	114	144	102	156	126
Hydrogen No., observed	111	137	115	148	117

* No hydrogen uptake.

Taken from HANAHAN, *J. Biol. Chem.* **211**, 313 (1954)

acid, palmitic acid-1-C^{14} and an unsaturated fatty acid, oleic acid-1-C^{14} into the lecithins of rat liver.[33] The same procedure as described above was followed, the lecithins were isolated, purified, and then subjected to enzymatic degradation in ether solution through the action of lecithinase *A*. The resulting free fatty acids and the lysolecithins were isolated, purified, and counted for their radio activity and analysed for the various elementary constituents. The data obtained in the liver experiments are shown in Table 2 and indicate very clearly that there is a distinct difference in the manner in which a saturated fatty acid and an unsaturated fatty acid are handled insofar as their incorporation into the lecithin molecule of the liver. These data support strongly the proposal that there is a metabolic as well as a positional asymmetry to the fatty acids of these lecithins. Among the possible conclusions which may be drawn from these data is that the β position of the lecithin molecule is concerned mainly with the metabolism of the saturated fatty acids whereas the α' ester position is involved only in the metabolism of the unsaturated fatty acids. This proposal is supported also by the observations that in all of the experiments reported here the assay of the total amount of label in the saturated and unsaturated fatty

acids by an oxidative technique[6] showed that all the acids in the β position were saturated and all the acids in the α' position were unsaturated. This confirmed by a different technique the previous observation to the positional asymmetry of the fatty acids in the liver lecithins[29] and also shows that this asymmetry can extend to the lecithins in the intestinal tract and the lymph. In a consideration of all possible interpretations of the results, one must not rule out the possibility that there is a distinct difference in the rate of synthesis and degradation of the two ester groups, for example the α' ester might be the

Table 2. Distribution of C^{14} in the α' and β fatty acids of liver lecithins of rats fed palmitic acid-1-C^{14} and oleic acid-1-C^{14}

In each experimental group, a total of four rats were used and were administered either 1×10^6 c/m Pal-1-C^{14} or Ol-1-C^{14} (per animal) in 0·5 ml olive oil
(Ref. 33)

Exp. No.	*Traction**	*Palmitic acid-1-C^{14}*			*Oleic acid-1-C^{14}*		
		Total c/m	*c/m/mg Fatty acid*	*% in*	*Total c/m*	*c/m/mg Fatty acid*	*% in*
I	FFA (α')	5,150	134	9	1366	44	44
	LL (β)	44,550	1400	91	1400	55	56
	Lecithin	49,000	742		1775	50	
II	FFA (α')	1,500	21	6	1200	25	53
	LL (β)	17,250	300	94	1375	22	47
	Lecithin	20,550	165		2700	21	

* FFA = free fatty acid.
LL = lysolecithin.

more reactive than the β ester group and consequently be metabolized or turned over at a much different rate. This may be formulated as follows:

$$\begin{array}{l} \qquad\qquad\qquad\qquad\quad CH_2O \leftrightarrow \text{Metabolically active acyl group} \\ \qquad\qquad\qquad\qquad\quad | \\ \text{Metabolically stable acyl group} \rightarrow OCH \\ \qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad\quad CH_2O \quad PC \end{array}$$

Thus it is evident from these remarks that lecithinase *A* as well as the other lecithinases may prove very useful in elaborating the role in which phospholipides and other complex and simple lipides are metabolized in the body.

4. *Purification and properties*

In the reports cited below the primary assay system for lecithinase *A* had been the neurotoxic effect or haemolytic activity of the particular component, while no attention had been directed toward a detailed study of the activity of these fractions on lecithin and hence definitive establishment of the crystalline compound *A* as pure lecithinase *A*. In fact in none of the studies cited below on crystalline material has any correlation been made between the increase in

purity of the protein with an increase in "lecithinase *A*" activity on well-defined substrates coupled with a suitable identification of the products. Although the data are very suggestive as to the identity of lecithinase *A* with this material, it would appear most desirable to be able to firmly establish this point.

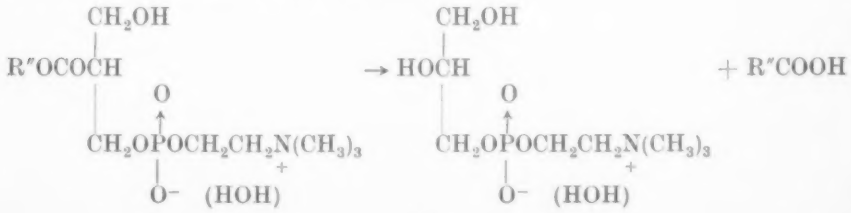
SLOTTA and FRAENKEL-CONRAT reported the isolation of the neurotoxic principle, crotoxin, of *Crotalus terrificus terrificus*.[66] By means of heat treatment, ammonium sulphate fractionation and crystallization from pyridine acetate solutions, crystalline crotoxin was obtained and this was later shown electrophoretically and ultracentrifugally homogeneous.[52] Only brief mention is made of a lecithinase value for this material, but no data or evidence is presented as to the derivation of this term. GHOSH and DE[25] claim that the neurotoxin of this venom can be partially separated from the heamolysin and, therefore contended that the crystalline substance obtained by SLOTTA and FRAENKEL-CONRAT was a mixture of at least two proteins.

DE[17] prepared a highly purified haemolysin fraction of the venoms of *Naja tripudians* and *Bungarus fasciatus* and crystallized these components after a systematic precipitation, adsorption, and elution process. By the criteria employed in solubility determinations and cataphoresis, it was reported that homogeneous components were obtained. No data on its activity on lecithin substrates were reported.

In their studies on the lecithinases in bacteria, HAYAISHI and KORNBERG[34] reported the isolation of active lecithinase *A* fractions. Specifically, their technique involved rupture of the cells with alumina paste, extraction of active enzyme with alkaline buffer, and separation of the enzyme by differential centrifugation, into two fractions, residue (*R*) and soluble (*S*). Both *R* and *S* were necessary for degradation of egg lecithin but either *R* or *S* alone attacked yeast lecithin and together gave an additive effect. On the other hand, dimyristoyl lecithin was attacked by fraction *S* but only slightly by fraction *R*. There appeared to be a requirement of calcium ion for fraction *R* activity. Fraction *S* could be replaced to a limited extent by ferrous ion. One possible interpretation of these data is that perhaps more than one type of lecithinase *A* activity, for example, one necessary for unsaturated and one necessary for a saturated compound, is present.

B. Lysolecithinase B

This enzyme can specifically effect the hydrolytic cleavage of the ester group on the β position of a lysolecithin (β-acyl-L-α-glycerylphosporylcholine) with release of a free fatty acid and glycerylphosphorylcholine.



In a consideration of enzymatic attack at the β-ester group of either lecithin or lysolecithin, one is confronted by seemingly conflicting evidence. In the enzymatic activity in extracts of aged rice bran and similar extracts from the mycelia of *Aspergillus oryzae*, CONTARDI and ERCOLI[14] reported that fractionation of the extracts into lecithinase *A* and lecithinase *B* resulted in a lecithinase *B* fraction which also attacked lecithin. Since their preparations were relatively crude lead acetate precipitates, there is a strong possibility that their enzyme preparation was contaminated with lecithinase *A*. LE BRETON and PANTALEON[47] also reported that they observed the degradation of lecithin by lecithinase *B* in crude extracts of plasma and pancreatic juice. However, again, the inhomogeneity of their preparations cast doubt on the existence of such an enzyme. Thus, on the basis of the evidence to be cited below, there is strong support for the proposal that only a lysolecithinase *B* and no lecithinase *B* exists in nature. Furthermore, the activity attributed to a lecithinase *B* must be considered to have been derived from a mixture of a lecithinase *A* and a lysolecithinase *B*.

1. *General remarks on reaction*

Lysolecithinase *B* activity has been reported in a variety of sources, but the evidence in general for its presence was based on the loss in haemolytic activity of the reaction mixture. In fact, it has been only within the past ten years that the exact proof for the chemical nature of the products of this reaction have been forthcoming. The presence of this enzyme has been clearly shown in *P. notatum*,[23, 70] pancreas,[64] and *Serratia plymuthicum*.[34] It is of interest that this enzyme is present in very high concentration in both the mould and the bacteria, while the other enzymes such as lecithinase *A* and *C* are present at a much lower concentration or else are completely absent. It would appear that this enzyme may be present for the specific purpose of removing rapidly from the cellular environment any lysolecithin which may be formed.

As has been mentioned previously, lecithinase *A* (as well as *C* and *D*) is influenced considerably in its reactivity by diethyl ether, but lysolecithinases appear to be unaffected in activity by this solvent. In the latter case, the substrate is easily soluble in water, while with the former enzyme systems, all the substrates exist as emulsions in aqueous reaction systems and hence the active site of attack may not be as easily accessible to the enzyme.

2. *Reaction systems and substrate specificity*

In this enzymatic reaction, wherein free fatty acid and glycerylphosphorylcholine are the products, one is presented with several possible ways with which to follow the course of degradation. Among the techniques which have been most widely used are: titrimetric assay for free fatty acid, change in the ester content by hydroxamic acid reaction, determination of residual lysolecithin by the triiodide method, and assay for the glycerylphosphorylcholine formed.

FAIRBAIRN[23] found that *Penicillium notatum* was an excellent source for lysolecithinase *B*. In his procedure the dried mat was pulverized with sand and

autolysed in water under toluene for 24 hr at room temperature. After filtration and washing, the combined filtrates were dialysed at 4° against running distilled water. After dialysis, either of two procedures could be utilized: (1) the extract was diluted with water and stored at 4° under toluene; (2) the solution in the dialysis sack was suspended in glycerol. He observed that aqueous extracts lost 80 per cent of their activity at 4° in two and a half months while glycerol preparations remained at essentially the same activity.

The test system consisted of a mixture of the lysophospholipides prepared from egg yolks, which contained approximately 20 per cent lysocephalin and 80 per cent lysolecithin and were completely saturated. All components were dissolved in veranol-acetate buffer, *p*H 4·0 and incubated at 30°. The original solution was clear, but as the enzyme reaction proceeded, it became quite turbid, owing to liberation of insoluble fatty acids. It was observed that a satisfactorily complete separation of the lysophospholipides and fatty acids from glycerylphosphorylcholine could be made through the use of colloidal iron-magnesium sulphate method of FOLCH and VAN SLYKE.[24] The lysophospholipides in the precipitate were then determined by analysis for phosphorus. No attempt was made to identify glycerylphosphorylcholine but it was found that any added pure glycerylphosphorylcholine could be separated by this technique. The method was valid only in the virtual absence of enzymes liberating choline or inorganic phosphate, or both, from glycerylphosphorylcholine. FAIRBAIRN observed that the enzyme liberated phosphate only at very high enzyme concentrations and over very prolonged period of time. No activity of the lysolecithinase *B* on lecithins or cephalins was apparent, but inasmuch as the mixture contained lysocephalins there was evidence that they were also attacked in this reaction system. When the *p*H of the reaction was 3·8 to 4·4, the maximum reactivity was no greater than 31 per cent, which could be correlated with a significant gel formation in the reaction system. Cyanide at 0·01 M completely inhibited the enzyme. Addition of fatty acids to the system had very slight effect while added glycerylphosphorylcholine had no effect at all.

SHAPIRO[64] has reported that there are present in acetone powders of ox pancreas two different enzyme systems which catalyse a formation of glycerylphosphorylcholine. One of the enzymes, a lysolecithinase, catalyses the hydrolytic splitting of lysolecithin and is without effect on lecithin. The second system splits lecithin into GPC without any concomitant decrease in the total ester content, suggesting the transfer of the fatty acids to an unknown carrier. SHAPIRO has elaborated on the former system, but the latter system has not been described further. In his system, SHAPIRO used the acyl ester content as one of his assay systems and another one was the determination of free choline formed by acid hydrolysis from the enzymatically liberated glycerylphosphorylcholine. In the presence of glycerol the time activity curve was linear at least up to 50 per cent of cleavage of the total substrate. Without glycerol, the initial rate of reaction was normal, but the activity disappeared after a short time. SHAPIRO considered that the glycerol stabilized the enzyme. Calcium,

magnesium or potassium, or cysteine, had no effect on the reaction. This lysolecithinase *B* preparation had no activity towards triolein or lecithin.

In a study of the lysolecithinases in the bacteria *Serratia plymuthicum* HAYAISHI and KORNBERG[34] followed the action of this enzyme in the following manner: the glycerylphosphorylcholine, liberated by lysolecithinase *B* activity, was further degraded by a GPC-diesterase preparation. The choline liberated in the latter reaction was assayed by the microiodide technique and used as an index of lysolecithinase *B* activity. This is a sensitive and specific technique but again poses the additional task of the preparation of the diesterase and the

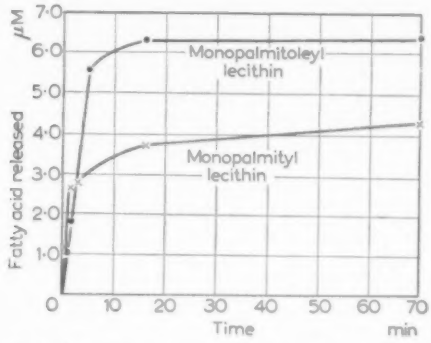


Fig. 5. Hydrolytic cleavage of lysolecithins by lysolecithinase *B*. Taken from UZIEL and HANAHAN. *J. Biol. Chem.* **220**, 1 (1956)

assurance each time that this preparation had no other lecithinase-like activity.

In the previously cited studies on lysolecithinase *B*, no isolation or unequivocal identification of the organic phosphate ester formed in the reaction was made, and in some cases the evidence presented could be interpreted as a formation of phosphorylcholine and a diglyceride. Hence, in a study designed to ascertain the structural requirements for a lysolecithinase *B* activity and the chemical nature of the products formed, UZIEL and HANAHAN[70] followed the action of a lysolecithinase *B* preparation from *Penicillium notatum* on pure unsaturated and saturated lysolecithins, β-monopalmitoleyl-*L*-α-glyceryl-phosphorylcholine and β-monopalmityl-*L*-α-glycerylphosphorylcholine, respectively.

The reaction system was composed of a mixture of the lysolecithin and enzyme in 10^{-4} M HCl and incubated at 30°. Inasmuch as only fatty acids and glycerylphosphorylcholine were released, the enzymatic activity could be followed either by titration of the free fatty acids or by colorimetric assay of the unreacted lysolecithin. In the former assay system which was used in all rate studies, the procedure required only the addition of freshly boiled ethanol and the free fatty acids could be titrated with dilute sodium hydroxide in 90 per cent methanol. In this type of system, distinct differences were noted in the reactivity of the unsaturated as compared with the saturated lysolecithin (Fig. 5). The reaction system with the unsaturated lysolecithin formed an emulsion at first which later separated into two layers with an emulsion at the

interface. In general, the reaction proceeded very smoothly and depending on enzyme concentration could be carried to completion. On the other hand, a decidedly different type reaction appeared to occur with the saturated lysolecithin. With this substrate the reaction would normally only proceed at the most to 50 per cent completion and the mixture always formed a thick gel. This is in agreement with the observations of FAIRBAIRN.[23] The reason for this gel formation is the simultaneous action of another enzyme, which will be discussed later and is apparently an isomerase. A typical titration curve for the action of lysolecithinase *B* on this saturated substrate is shown in Fig. 5.

Another assay technique which was very useful in preparative runs involved the colorimetric assay of the unreacted lysolecithin by the tri-iodide method.[3] This was based on the observation that neither glycerylphosphorylcholine nor phosphorylcholine formed an insoluble periodide with potassium tri-iodide, while lysolecithin in 1N HCl is insoluble[34] and these observations were adapted to give an analytically useful technique.[70] The resultant products of the reaction, *L*-α-GPC and free fatty acid were isolated and identified. On a preparative scale *L*-α-GPC could be prepared and isolated in analytically pure form as a cadmium salt. In a typical system the unsaturated lysolecithin and sufficient enzyme to cause greater than 95 per cent hydrolysis were incubated in 10^{-4} M HCl at 30° for 3½ hr. The milky mixture was extracted with diethyl ether and the aqueous phase, carefully neutralized and concentrated to a small volume. Repeated treatment with absolute alcohol removed any protein, and the extracts were concentrated to a syrup and then finally extracted with chloroform. This removed any residual lysolecithin and allowed isolation of the *L*-α-GPC as a cadmium salt from the chloroform insoluble fraction. The yields of pure GPC were of the order of 40–50 per cent. Comparison of its properties with those reported by BAER and KATES[4] showed to be identical with their compound (Table 3).

Table 3. Composition of enzymatically formed glyceryphosphorylcholine (isolated as crystalline cadmium salt)

	Found	*Theory**
P, %	6·20	6·28
N, %	2·92	2·84
Choline, %	26·0	24·5
Cadmium, %	23·2	22·7
Vic glycol/P	0·99	0·99
$[\alpha]_D^{25}$	− 1·36°	− 1·4° ± 0·1°
Melting point†	Sinter 95–96° Meniscus 168–170°	Sinter 97° Melts 100–102°

* Compared with the values obtained by BAER and KATES, *J. Amer. Chem. Soc.* **70**, 1394 (1948).

† In discussion, Dr. KATES suggested that the difference in melting points may be resolved on the basis that the heating rate in one case is faster. BAER and KATES used a slower rate, and hence may have had a dehydrated sample. However, a completely dehydrated sample melted at 168–170, which was the same as the hydrated sample shown above.

Although the mould used for the preparation of the enzyme in this study was probably not of the same strain as that used by FAIRBAIRN,[23] the lysolecithinase *B* showed the same general characteristics: (1) no inorganic phosphate or free choline were formed during the reaction; (2) lecithin was not attacked by the enzyme in aqueous media from *p*H 4–7 or in diethyl ether. The *p*H optimum appeared to be near 4·0. It is of interest that bacterial lysolecithinase *B*[34] has its *p*H optimum near 6 as does the ox-pancreas lysolecithinase *B* of SHAPIRO.[64] Hence, to date, the main structural requirements for the substrate in this reaction are that it be a monoacyl substituted glycerylphosphorylcholine and although configurational specificity has not been investigated, most probably an *L*-α-configuration is necessary. On the basis of present evidence, there appears to be no necessity for the fatty acids to be of a single species, but it is of interest that the *P. notatum* system attacks an unsaturated lysolecithin much more rapidly and to a larger extent than a saturated lysolecithin.

These apparent differences, however, may be attributable to the following reasons: (1) the enzyme is specific for the unsaturated lysolecithin and the saturated analog is hydrolysed due to a similarity in structure; (2) the enzyme preparation is composed of more than one type of lysolecithinase *B* or enzymes active toward this substrate; (3) the formation of the gel in the saturated system may remove the substrate and/or enzyme from solution and thus reduce the rate of hydrolysis.

3. *Isolation of enzymes*

Lysolecithinase *B* has been prepared in a highly purified form from ox pancreas by SHAPIRO[64] and in partially purified form from bacteria by HAYAISHI and KORNBERG[34] and from a mould by FAIRBAIRN[23] and by UZIEL and HANAHAN.[70]

When an acetone powder of ox pancreas was used as a source, SHAPIRO was able to prepare a crystalline form of lysolecithinase *B*. In this procedure the enzyme was extracted by a glycerol-sodium bicarbonate extraction, which was then adjusted to *p*H 5 and the active precipitate dissolved in phosphate buffer. Subsequent fractionation with ammonium sulphate yielded in the 25–28 per cent fraction an active crystalline enzyme preparation. This procedure gave a twenty-five-fold purification, based on activity towards lysolecithin, with a yield of 30–35 per cent. A purification of forty-five-fold could be accomplished, but only with a considerable loss in material. As SHAPIRO emphasizes, it remains to be ascertained whether this was a homogeneous enzyme or not but his data, wherein there is an increase in activity with crystallization, at least supports the conclusion that it is indeed a lysolecithinase *B* and not a contaminate. It is of interest that UZIEL and HANAHAN[70] have found no lysolecithinase activity in commercial pancreatin or fresh rat pancreas, which may indicate a species specificity.

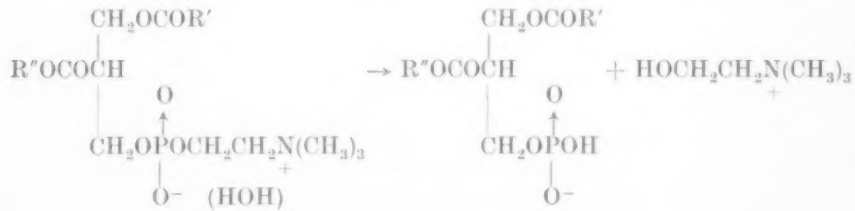
Utilizing the same techniques as described in their preparation of lecithinase *A* from *Serratia plymuthicum*, HAYAISHI and KORNBERG[34] found that the lysolecithinase *B* activity was distributed both in the residue and the soluble

fraction, with a higher specific activity enzyme in the residue. They observed that the lysolecithinase *B* activity was by far the most potent of the series of enzymes for lecithin degradation in this bacteria. By their calculations, the specific activity of this enzyme was almost fifteen times that of lecithinase *A*. However, it is well to point out that this may not represent a real difference, due to the fact that the substrates in each case may not have been assayed under optimal physical conditions. Whereas it is a completely homogeneous soluble substrate for the lysolecithinase *B* action, the substrate in the lecithinase *A* assay is an emulsion (micellar form) which may make a fewer number of the sites of attacks available to the enzyme, and consequently affect the actual specific activity value.

A modification of the procedure of FAIRBAIRN[23] can yield a very active, stable preparation from *P. notatum*.[70] In this technique, the dry mycelia are homogenized in distilled water in a Waring blender for ½–1 min. The mixture is then incubated under toluene for 24 hr at room temperature. The contents are then centrifuged for 20 min at 2000 r.p.m. The supernatant is decanted, filtered, and the clear red-brown filtrate cooled to 0° and saturated with approximately 70 gm of ammonium sulphate. The precipitate is discarded and the resultant supernatant fluid is dialysed against water until the solution is only faintly coloured. The preparation is lyophilized for storage. If further purification is desired, the solution after dialysis is decolourized by Darco-G-60 acid-washed charcoal. The charcoal is removed by centrifugation and/or filtration. The clear, coloured fluid is lyophilized and the powder is stored in a desiccator, and is stable for an indefinite period. One milligram of this powder can degrade 10 mg of unsaturated lysolecithin to 90 per cent completion in 3 min.

C. Lecithinase C

This enzyme can cause the splitting of the nitrogen base-phosphoric acid ester bond of lecithin, with the release of free choline and the production of a phosphatidic acid.



On considering the source of various types of lecithinases, it is of considerable interest to note that lecithinase *C* has been found only in plants and never detected to date in any mammalian tissue. At the present time the reason for this enzyme being present in such a high concentration in the carrot or cabbage, for example, is inexplicable, just as is the presence of only a lysolecithinase *B* in the mould, *P. notatum*.

1. *Primary mode of attack*

In the first evidence presented for the existence of this enzyme, YOSINAGA[74] reported the release of choline from lecithin by the action of takadiatase extracts. In his system, the egg-yolk lecithin was mixed with the enzyme and buffer at *p*H 4·5 and incubated for 24–144 hr at 37°. Although calculations of the amount of choline released in his system were difficult to make from the data presented, it appeared that in some 144 hr only 2–3 per cent of the total choline was released. At best it represented a very weak lecithinase *C* activity and cannot be construed as strong evidence for its occurrence.

In a study designed to investigate the chemical nature of the phospholipides of the carrot and the cabbage, HANAHAN and CHAIKOFF[26, 27] observed that the isolated lipides in repeated instances had a low N/P ratio. When the carrots or

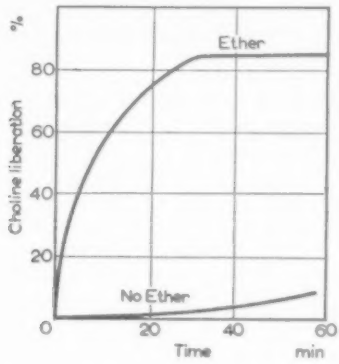


Fig. 6. Influence of diethyl ether on the enzymatic liberation of choline from lecithin by spinach chloroplasts.
Taken from KATES M. *Nature* **172**, 814 (1953).
Ref. 35

the cabbage leaves were steam treated prior to isolation of the lipides, the isolated phospholipides had an N/P ratio near the expected value for such compounds. This indicated strongly that there was an enzymatic degradation occurring in the course of the isolation of the lipides and that it was a lecithinase *C* type of activity. As has been suggested,[28] this type of enzymatic activity most probably accounts for the observation of CHIBNALL and CHANNON[11, 12] on the isolation of a phosphatidic acid from cabbage leaves. When the enzyme from carrot and cabbage leaves was examined more closely in an isolated system, it was observed that the lecithinase *C* was apparently the only type of lecithinase present. A typical reaction system consisted of a soybean lecithin emulsion plus the enzyme and buffer at a *p*H from 5·1–5·9, with incubation at 25°. Usually, within 2–3 hr some 30–40 per cent of the theoretical amount of the nitrogenous base had been liberated. The mixture was extracted with ether and the aqueous and ether fractions analysed for choline and nitrogen. The loss of ether-soluble nitrogen was used as an index of activity. It was evident

from the data that more nitrogen was liberated than could be accounted for by the choline and thus other types of phospholipides must have been attacked by this enzyme system.

KATES[35, 36, 37] has investigated the mode of action of the lecithinases of various plants. In particular, he has focused attention on the enzymes present in the sugar beet, spinach, cabbage leaves, and carrot root. It is of considerable interest that KATES observed the activating effect of diethyl ether on the release of choline from lecithin by the lecithinase *C* of these tissues.[35] Although

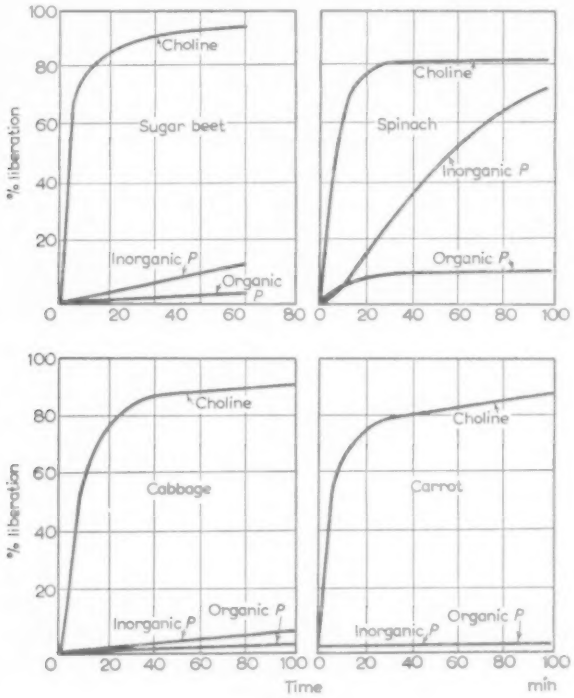


Fig. 7. Kinetics of liberation of choline, inorganic P, and water soluble organic P from lecithins by plastids.
Taken from KATES M. *Canad. J. Biochem. Physiol.* **33**, 575 (1955).
Ref. 37

the reaction does not proceed in an ether solution as does the lecithinase *A*, the activating effect of this solvent was quite pronounced as shown in Fig. 6. In this preliminary communication the choline-liberating activities of the juices from spinach, sugar beets, or cabbage leaves were associated entirely with the chloroplast fraction. The cytoplasmic fractions were inactive and were inhibitory in most cases. These findings could explain the reported absence of lecithinase *C* in spinach press juice,[1] which corresponds to the cytoplasmic fractions. The observation that chloroplast enzymatic action is activated by ether supports the

explanation of the origin of phosphatidic acids in the ether extraction of cabbage cytoplasm and emphasizes the cautious use of this solvent in lipide extractions.[35]

Later, KATES[36, 37] was concerned with the identification of the water-soluble organic phosphates formed by plant plastid enzymes and the kinetics of these systems with reference to the sequence of degradation steps. The enzyme assay system consisted of egg lecithin, buffer, and enzyme at the desired *p*H, which were then shaken with diethyl ether and incubated at 25° for a suitable period of time. After the addition of TCA or perchloric acid, the mixture was extracted with diethyl ether and the aqueous and ether soluble phases analysed for free choline, total phosphorus, and free phosphorus.

Time course curves for the liberation of choline, inorganic phosphate, and and water-soluble organic phosphorus from egg lecithin by sugar beet, spinach, cabbage or carrot plastids are shown in Fig. 7. In this system, the liberation of choline was rapid and yielded more than 80 per cent of theory in 1 hr. The values for the first-order reaction constants were quite constant up to approximately 70 per cent liberation but increased rapidly thereafter. It was observed that the liberation of inorganic phosphate, though very small in relation to the total choline release in some of the tissues, nevertheless did occur, particularly in the spinach and sugar beet. On the basis of the time activity curves and the *p*H effect on the liberation of choline and inorganic phosphate KATES concluded that the primary occurrence was the liberation of choline and subsequently any removal of the phosphate would be derived from the phosphatidic acid formed from the first reaction. In addition, no action of spinach chloroplast on phosphorylcholine, lysolecithin, or glycerylphosphorylcholine was observed under optimum condition.

In addition to the enzyme studies of this investigation, KATES[37] also isolated and identified the liberated phosphatidic acid. The most profitable avenue of approach was to use carrot root chloroplast, inasmuch as cabbage or spinach yielded the chlorophyl pigments which were unwieldly contaminants. The analysis of the free acid showed it to have the expected composition and to be of the *L*-α-configuration. This procedure allowed a very smooth and convenient route to a naturally-occurring phosphatidic acid.

ACKER *et al.*[1] reported that carrot press juice liberated inorganic phosphate as well as choline from yeast lecithin, and in addition could hydrolyse phosphorylcholine. They speculated that phosphorylcholine may be cleaved first from lecithin, and then degradation of the phosphorylcholine to inorganic phosphate and choline may explain the results. However, the amounts of free choline were far greater than the inorganic phosphate and hence in agreement with the above investigations, they also concluded that choline was liberated first, with any inorganic phosphate arising most probably from degradation of the resultant phosphatidic acid. DAVIDSON *et al.*[16] used an aqueous cabbage leaf lecithinase *C* preparation free from chloroplast, and found that it degraded synthetic *L*-α-lecithins (saturated) to give a theoretical yield of choline, while lysolecithins and synthetic β-lecithins were attacked to a partial degree. Sphingomyelin was unaffected.

2. *Activation of enzyme and substrate requirements*

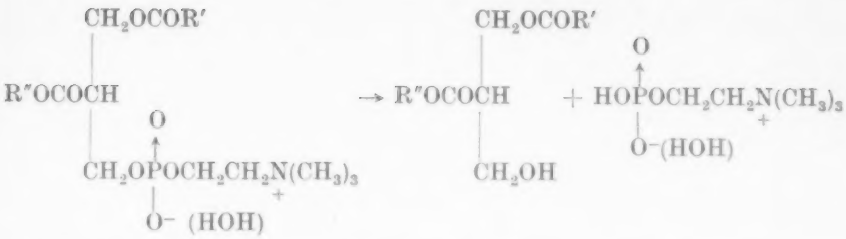
Recently, KATES[38] has investigated in detail the mechanism of the ether stimulation of the plastid lecithinase and the effect of various solvents and surface active agents on this enzyme. When observed microscopically, diethyl ether produced a coalescence of the chloroplasts and lecithin. Evidence for a complex formation between lecithin and the chloroplasts under the influence of the ether was also obtained. During the reaction the enzyme remained bound to the particulate matter. Besides ethyl ether, the following solvents produced comparable stimulation effects: diethyl ketone, dipropyl ketone, ethyl propionate, and ethyl butyrate. On the other hand, alcohols, aldehydes, cyclic ethers, cyclic ketones, lactones, hydrocarbons, and halogenated compounds produced little or no stimulation. Anionic detergents produced large stimulations within a limited concentration range while nonionic and cationic detergents showed no activating effect. Methanol, at 20 per cent concentration, further increased the ether stimulation by approximately 30 per cent. This latter solvent also increases the stimulation with the less active stimulators, such as petroleum ether and benzene, to values comparable to those of ethyl ether.

Further evidence has also been supplied by KATES[38] on the specificity of attack of this enzyme on various substrates. Glycerylphosphorylcholine, lysolecithin, and glycol lecithin are not hydrolysed by cabbage chloroplasts, either in the presence or absence of ether. However, dimyristoyl lecithin, distearoyl lecithin, and dipalmitoleyl lecithin were hydrolysed in the presence of ether but not in its absence. With the saturated substrates the rate of hydrolysis increased with decreasing fatty acid chain length. Synthetic dimyristoyl phosphatidyl ethanolamine was hydrolysed in the presence of ether but not in its absence, while synthetic distearoyl phosphatidyl serine was attacked very slowly in the presence of ether, but not in its absence. Both the lecithin and cephalin components of soybean phosphatides were hydrolysed in the absence of ether, but the rates were increased in the presence of the ether. Since a mixture of synthetic cephalin and yeast lecithin was not hydrolysed in the absence of ether, KATES concluded that soybean phosphatide must contain a natural activator.

Recently, TOOKEY and BALLS[68] have investigated the action of a lecithinase *C* from cabbage leaves and cottonseed meal. The substrate for this reaction was a "purified" soybean lecithin which contained cephalin, lecithin, inositol phospholipides, glycerides, and glucosides. For the first time a soluble preparation of this enzyme could be made with cottonseed meal as the source, whereas the cabbage enzyme was localized in an insoluble fraction. Further, the soluble enzyme preparation was not activated by diethyl ether, while the cabbage system was stimulated by this solvent. On a comparative basis, both enzyme preparations released only the nitrogen base from the phospholipide substrate.

D. Lecithinase D

This enzyme specifically effects the hydrolytic rupture of the phosphorylcholine from lecithin with the concomitant formation of an α, β-diglyceride.



Perhaps one of the most specific enzymes in the lecithinase series is lecithinase *D*. Insofar as can be ascertained, it exerts a high specificity for the lecithin molecule, with little or no activity toward compounds such as lysolecithin, phosphatidyl ethanolamine, glycerylphosphorylcholine, and the sphingomyelins. Until recently this enzyme had been found only in bacterial systems, but evidence presented by KATES shows that they exist also in plants as well.

1. *General considerations and specificity of action*

In 1941, MACFARLANE and KNIGHT[56] reported on their findings that the α-toxin, the lethal haemolytic factor in *Cl. welchii* toxins, appeared to be identical with the lecithinase present. Moreover, it was established that this lecithinase could attack lecithin with the formation of only a diglyceride and phosphoryl choline (PC). In addition, the products of the reaction were isolated and identified as such. In this system the mixed phospholipides of egg yolk were emulsified in water and incubated at 37° with the toxin plus calcium ions at *p*H 7·4. During the course of reaction, a precipitate formed (diglyceride) and the mixture became more acid (PC formation) and thus frequent addition of dilute alkali was necessary to maintain the reaction. At the end of the incubation period, the reaction mixture was extracted with diethyl ether and the two phases analyzed for diglyceride and phosphorylcholine. Phosphorylcholine was isolated from the aqueous fraction as a calcium salt. The enzyme was active over *p*H range from 5–9 with an optimum in a broad range from 7–7·6. It required the presence of calcium ion for maximum activity. In addition this enzyme exhibited, as do many of the bacterial toxins, a relatively high stability to heat.

Later, CHU[13] showed that the lecithinases in the *Bacillus cereus* and *Bacillus mycoides* attacked lecithins and caused formation of phosphorylcholine and a diglyceride. They appeared to be identical with the *Cl. welchii* α-toxin. They were activated by calcium and magnesium ions but inhibited by sodium, potassium, ammonium, ferric, and aluminium ions. In addition, they exhibited a stability to heat. As with the *Cl. welchii* α-toxin, normal sera of a variety of animals strongly inhibited the action of the *Bacillus cereus* lecithinase *D*. CHU reports that the *Bacillus cereus* lecithinase *D* exerted an activity toward cephalin. However, there was a paucity of evidence to support this conclusion. In their initial work, MACFARLANE and KNIGHT used the mixed phospholipides of egg yolk, which undoubtedly contained some cephalins but their evidence based on the usual composition of these crude phospholipide fractions, would lead one to consider that only lecithin was attacked. More detailed information

on this point seems necessary, but it is evident from the work of various investigators that it is highly unlikely that the cephalin fraction is attacked by lecithinase *D*.

In a study on the lecithinase of *Cl. bifermentans* toxin, LEWIS and MACFARLANE[51] concluded that the same type of lecithinase was present in this α-toxin as in the α-toxin of *Cl. welchii*. This confirmed the preliminary results reported by MILES and MILES[58] on occurrence of this enzyme in the *Cl. bifermentans* toxin. LEWIS and MACFARLANE found no phosphodiesterase or lipase activity in their preparations. From these studies it was concluded that this lecithinase was a typical lecithinase *D* and hence attacked lecithin with formation of a diglyceride and phosphorylcholine.

Inasmuch as phosphorylcholine, $pK_2 = 5{\cdot}6$, should liberate CO_2 from a buffer mixture, ZAMECNIK *et al.*,[75] found it feasible to follow this action of lecithinase *D* on lecithin by a manometric technique. In comparison with the acid-soluble phosphorus technique of MACFARLANE and KNIGHT they found that this manometric technique was very useful and reproducible. They found their lecithinase *D* preparation to be completely inactive towards phosphatidyl serine, sphingomyelins, ox-brain cerebrosides, glycerylphosphorylcholine, and soybean phospholipides. They presented indirect evidence for the inactivity of this enzyme on lysolecithin. A requirement of either calcium ion or magnesium ion was indicated. On the basis of the success with this particular technique they applied the specificity of the toxin lecithinase *D* to a study of the toxin-antitoxin reactions and observed a competition between antitoxin and substrate for the enzyme-toxin. They found that the combination of the antitoxin with toxin is enormously inhibited by the presence of lecithin. It appeared from their results that lecithin and antitoxin compete specifically for combination with the same reactive site on the enzyme molecule. Thus, these findings provided a partial explanation for lack of the effectiveness of antitoxin when given late in the course of *Cl. welchii* infection. The lecithin toxin combination is strong but can be altered by the addition of excess of antitoxin.

HANAHAN and VERCAMER[32] found that the lecithinase *D* of the toxic filtrate of *Cl. perfringens* (welchii) type *A* cultures could attack lecithin in solvents systems such as diethyl ether or 98 per cent diethyl ether—2 per cent ethanol. It again provided an example of an enzyme-substrate complex since the enzyme *per se* was not soluble in these solvents. The use of this type of system allowed a comparatively reasonable approach to a kinetic study of this system, for the reaction can be followed titrimetrically in this solvent and the products of the reaction, phosphorylcholine and diglyceride, respectively, are easily separated by differences in ether solubility. The use of a fully unsaturated lecithin, (dipalmitoleyl)-*L*-α-lecithin and a fully saturated lecithin, (dipalmityl)-*L*-α-lecithin showed both substrates to be attacked by this enzyme system. Consequently it provided a good route to the correspondingly pure diglycerides. As noted previously for this enzyme[56] calcium ion was required for optimum conditions. No action of lecithinase *D* on monopalmitoleyl-*L*-α-glycerylphosphorylcholine, monopalmityl-*L*-α-glycerylphosphorylcholine or yeast cepha-

lin was noted. Long and McGuire[53] have reported that lecithinase *D* acts as expected on egg lecithin but exhibited no activity toward saturated lecithins in an aqueous medium. Perhaps it is more a case of the difficulty of effectively emulsifying the saturated lecithin in water, rather than a specificity of action.

Although the presence of lecithinase *D* in bacterial toxins has been well established, its existence in higher plants was only recently shown by Kates.[37] In particular, it was observed that spinach chloroplast enzymes could cleave phosphorylcholine from lecithin. Phosphorylcholine was identified by paper chromatography, but no evidence was presented on the diglyceride formed. As with the bacterial lecithinase *D*, the plastid enzymes did not attack lysolecithin.

2. *Purification procedures*

Only a limited success has been achieved in the area of purification of the lecithinase *D*. In particular it has been directed toward a purification of the α-toxin of *Cl. welchii*.

Van Heyningen[71] purified the α-toxin to some extent by absorbing it on calcium phosphate in the presence of acetone. An elution of this precipitate with 20 per cent saturated ammonium sulphate gave an eluate which contained 50–60 per cent of the toxin and was considered to have been purified approximately ten-fold. Subsequently van Heyningen and Bidwell[72] described procedures for purification of the α-toxin, which, though the yields or degree of purification were no greater than in the above technique, allowed use of larger volumes of culture filtrates. By use of ammonium sulphate fractionation alone, a fifty-fold overall purification of the α-toxin could be gained. Through the additional use of nucleic acid precipitation, a further five-fold purification could be achieved. In part, the toxin was estimated by measuring its combining power with antitoxin using lecithin as the indicator for slight excess of toxin. No information on the enzymatic activity of these fractions on purified substrates was supplied.

Roth and Pillemer[63] investigated the separation of the α-toxin from the other toxins in the filtrates from *Cl. welchii* type cultures. This could be accomplished by fractionation in methanol-water mixtures under controlled *p*H, ionic strength, and temperature below 0°. On the basis of their assay system, a 130-fold purification was achieved. When the results of the lecithinase combining power, lethal activity and alpha (hot-cold) haemolysin activity were correlated, they believed that only one component was responsible for all these activities. However, as they point out, the homogeneity of the protein must be established first before any conclusive statements can be made.

Again it would be of considerable interest to be able to associate these purification procedures with an increase in the specific activity of the enzyme towards a particular substrate.

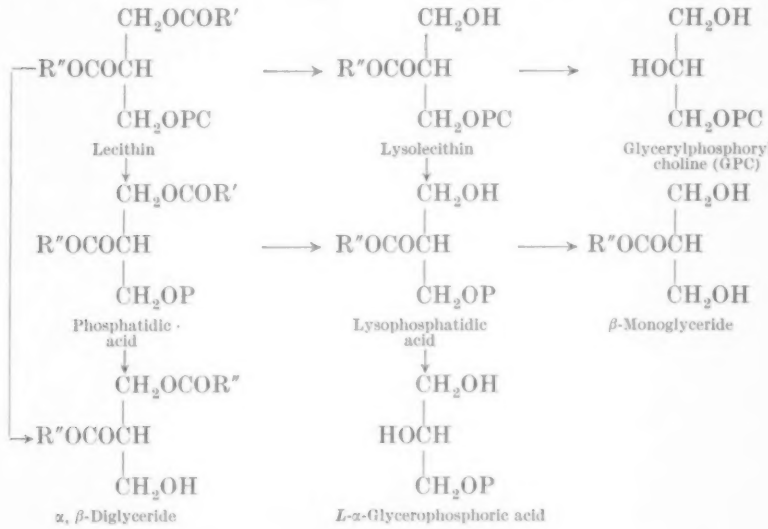
III. Other Possible Enzymes Involved in Lecithin Metabolism

When considering other possible sites of attack on the model compound, lecithin, one can envisage an equally important series of enzymes, other than

those previously mentioned, which can properly fit into a classification system for lipolytic enzymes. Typical examples of such enzymes are the hydrolytic, transferring, oxidative, and synthetic types. There is evidence that these enzymes do exist and are of obvious importance in the overall metabolism of phospholipides.

A. Hydrolytic type

It is becoming increasingly apparent that there can be present in various tissues phospholipide-attacking enzymes, which can cause hydrolytic changes other than those depicted in the classical group discussed in the previous sections. The following diagrammatic scheme is presented as a guide in illustrating this point. Only lecithin and lysolecithin are used as typical starting substrates in this series of reactions.



As is obvious in this scheme, certain of these enzymes have already been well established as to their action; i.e., lecithin to diglyceride (plus PC) lecithin to phosphatidic acid (plus free choline), lecithin to lysolecithin (plus free fatty acid), and lysolecithin to GPC (plus free fatty acid). Only recently has any evidence been produced to establish the existence of any of the other enzymes.

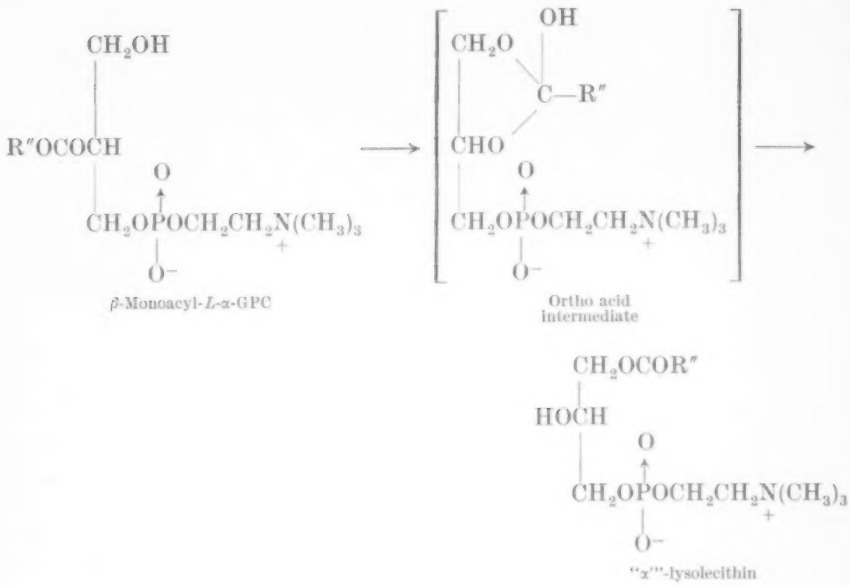
KATES[37] reported that the release of inorganic phosphate, through the action of spinach chloroplasts on lecithin, was significant, though much slower than the liberation of choline. On this basis the conclusion was that phosphatidic acid appeared to be the most probably substrate for this reaction. However, the degradation of phosphatidic acid through lysophosphatidic acid could yield glycerophosphoric acid which can then be degraded by a phosphatase to glycerol and inorganic phosphate. Notwithstanding this possibility it is still important that such an observation has been noted.

Although this scheme is speculative, it points out areas wherein further investigation is required. Nevertheless, based on the specificity of some of the aforementioned lecithinases, it will indeed not be unusual to learn that these enzymes do exist. Concomitant with such studies it is mandatory that well-defined substrates should be available and used and that adequate characterization of the products shall be presented before definitive conclusions can be reached as to the mode of the enzyme action.

B. Isomerase

As mentioned under lysolecithinase *B* action, the activity of this enzyme toward the saturated lysolecithin was far less than towards an unsaturated lysolecithin (Fig. 5). FAIRBAIRN[23] in his original report on this enzyme system used a saturated lysolecithin as substrate and stated that first order kinetics were simulated only under specific conditions, i.e. when sufficient enzyme was present so that the reaction was less than 40 per cent complete in 15 min at 30°. CONTARDI and ERCOLI[14] reported that extracts of aged rice bran and the mycelia of *Aspergillus oryzae* destroyed egg lysolecithin (as measured by the loss of haemolytic activity) and produced a water soluble organic phosphate which was not further identified. The yields of this compound, however, were low. If the reaction was allowed to continue for one week at 37° only 20 per cent of the original phosphorus is found as water soluble organic phosphate. During this period a heavy precipitate was formed which was neither lysolecithin (by haemolytic tests) nor lecithin (by lecithinase *A* action). They presumed that this compound was a salt of fatty acid and lysolecithin.

During the course of an investigation on the lysolecithinase *B* on pure saturated lysolecithin, UZIEL[69] noted that the reaction mixtures tended to form gels and in many instances, appeared to be composed of crystals. A more detailed examination showed that an actual enzymatic activity, other than the hydrolytic activity, was present in the lysolecithinase *B* preparations. This is now considered to be a lysolecithin isomerase, which can cause a migration of the acyl group on the β position of lysolecithin (resulting from lecithinase *A* action on lecithin) to the α' position. This enzymatic activity has been found in *P. notatum* and also in fresh pancreas (rat) and commercial pancreatin, which contains no lysolecithinase *B* activity. Hence, it does not appear to be lysolecithinase *B* which is causing the change. The migrated product, an α'-lysolecithin, has been isolated in a pure form and subjected to chemical studies. The only detectable changes are in solubility, melting point, and optical activity. The typical changes of β-monostearoyl-glycerylphosphorylcholine are recorded in Table 4. In addition the infra-red spectra of both α'- and β-monostearoyl-lysolecithin are essentially identical. Inasmuch as this enzymatic activity is found in extracts where no lysolecithinase *B* activity occurs, it may be reasoned that they are separate enzymes. In attempting to ascertain the general mechanism by which this transformation occurs, the most plausible scheme appears to be the following:



In this scheme, the formation of an intermediate orthoacid is required. Attempts to determine whether this orthoacid is formed have not been successful, but the lack of any significant change in the carbonyl band in the infra-red of the α′-lysolecithin would tend to rule out any orthoacid being present as the final

Table 4. Composition and physical properties of β-monostearoyl-L-α-GPC(β-MSL) and α′-monostearoyl-L-α-GPC(α′-MSL)

(Ref. 69)

Compound	*Melting point*	*Phosphorus,* %	*Nitrogen,* %	*Choline,* %	$[\alpha]_D^{25}$
β-MSL	257–258°	5·72	2·59	22·4	− 2·87°
α′-MSL	235–236°	5·75	2·54	22·3	− 3·78°

component. It is of interest that the α′-lysolecithin may be prepared by incubation of the β-monostearoyl lysolecithin in 0·05 normal HCl for 12 hr at room temperature. The same typical crystal formation occurs and the main product is α′-monostearoyl lysolecithin. The structure proof for this compound centres about the oxidative degradation through use of aqueous permanganate at room temperature (*cf.* Section 2, *A*, 2 above). When compared with the β-monostearoyl lysolecithin, α′-monostearoyl lysolecithin is approximately ten to fifteen times slower in its reaction with potassium permanganate and this can form the basis of an assay system.

An interesting point is the observation by FAIRBAIRN[23] and confirmed by

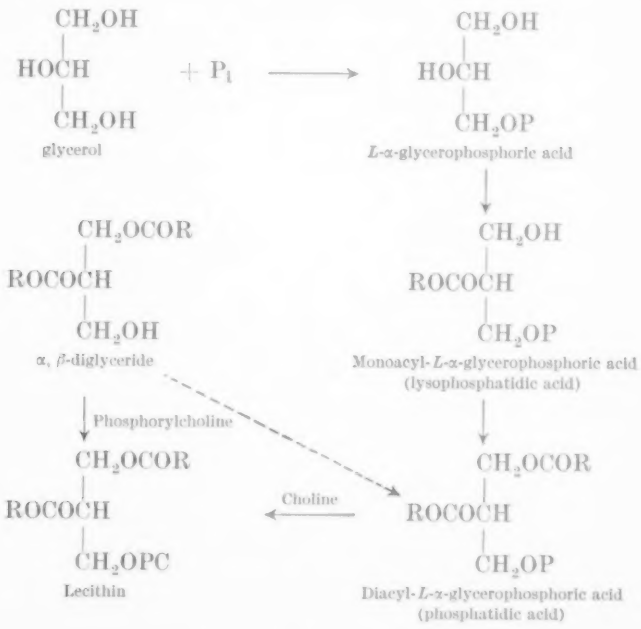
Uziel,[69] that cyanide is a potent inhibitor of this reaction. One possible explanation of this inhibition is that the orthoacid may be converted in part to a nitrile, which then effectively stops the acyl migration. Another possibility is the presence of a metal enzyme, which is cyanide sensitive.

When reasons for this type of enzymatic reaction are considered, several possibilities may be proposed. One is that this derivative may be the actual substrate for lysolecithinase *B* action. Another is that this derivative may present a possible pathway to a monoglyceride.

As is evident more definitive experimentation must be carried out in order to ascertain the probability of this latter type of reaction occurring in mammalian or plant tissues.

C. Synthetic enzymes

Within recent years, considerable progress has been made in the elucidation of the reactions leading to the synthesis of phospholipides. To a large degree, isolated systems have been used and attention has been directed toward the individual enzymes which might influence any of the reactions. One of the foremost problems in this type of study, other than the isolation of the enzymes involved, is the availability of suitable intermediates to use as test compounds, and adequate isolation and identification procedures for suspected products on a small scale. Nevertheless, on the basis of the data accumulated on this subject to date, the following series of reactions merit serious consideration as the routes of synthesis.



1. *Phosphate and glycerophosphate to lecithin*

In the initial phases of the synthesis of lecithin, KENNEDY[39] provided evidence that in the presence of active oxidative phosphorylation, rat-liver mitochondria could incorporate inorganic phosphate into the phosphorus-containing lipide fraction. In the absence of oxidative phosphorylation, no incorporation was evident. When isotopic compounds were used, it was observed that the specific activity of the final product was diluted by the addition of nonlabelled glycerophosphate. Glycerophosphate-P^{32} was vigorously incorporated into a phospholipide fraction. Only the α form of the glycerophosphate was active in this system and by means of chromatographic behaviour of the product on Dowex columns, KENNEDY concluded that the product was a phosphatidic acid. Subsequently, lecithin could arise by the reaction of choline with the phosphatidic acid.[41]

KORNBERG and PRICER[44] observed that by use of partially purified enzyme preparations from rat liver a synthesis of a phosphorus-containing lipide from *L*-α-glycerophosphate plus fatty acid could be achieved. The enzyme responsible for these transformations could be purified four-fold from homogenates by treatment with calcium phosphate gel or by fractionation with methanol at low temperatures. This system required ATP, coenzyme *A*, and stearic acid. Addition of choline, phosphorylcholine, glycerylphosphorylcholine, or glycerol did not affect the incorporation of the glycerophosphate. Two reaction products were tentatively identified by paper chromatography and were considered by these authors at that time to be monoacylphosphatidic (lyso) acid and the diacylphosphatidic acid. Later, these authors[45] reported that most probably monacyl derivative could have arisen through acid decomposition of the diacyl derivative and hence not represent an intermediate in the scheme. The instability of phosphatidic acid under mild conditions has been reported by OLLEY.[61] KORNBERG and PRICER[45] further found a very active esterification system present in the lyophilized guinea pig liver "residue" fraction. Using labelled fatty acids and radioactive phosphate, they found that *L*-α-GP could be esterified by two moles of higher fatty acids with this enzyme in the presence of ATP and coenzyme *A*. When the fatty acid-CoA derivative was substituted in the reaction system, no ATP or added free CoA was necessary. The product, which was considered to be a phosphatidic acid, was identified by its characteristics on Dowex columns and on the basis of the ester/P, C^{14}/P^{32} ratios. Unfortunately in the above studies sufficient material was not available to establish definitely the absence of any nitrogenous bases or indicate the possible dehydrogenation of the long chain acyl units.

2. *Phosphorylcholine conversion*

When one examines the other possible pathways of synthesis, the observations made by KORNBERG and PRICER[46] are of considerable interest. They reported that radioactive phosphorylcholine was incorporated apparently as a unit by rat liver or guinea pig liver extracts into an unidentified phosphorus-containing lipide and to a much higher extent than choline under similar conditions.

KENNEDY[40] studied a similar reaction in rat liver mitochondria which would incorporate choline into lecithin and required ATP but found that phosphorylcholine (PC) was inactive. On this basis, he concluded that PC was not involved in the synthetic pathway to lecithin.

On the other hand, in an investigation of the pronounced influence of phosphorylcholine on the oxidative metabolism of guinea pig liver mitochondria, RODBELL and HANAHAN[62] noted that phosphorylcholine was very vigorously incorporated as an intact unit into the phospholipide fraction, which was identified as lecithin and represented the only compound with detectable radioactivity. Both a pure diglyceride and phosphatidic acid stimulated the incorporation of phosphorylcholine into lecithin. Recently, KENNEDY and WEISS[42] reported that there must be two pathways involved in the biosynthesis of lecithin, one which involves choline and the other which utilizes phosphorylcholine. This was based on the finding that cytidine triphosphate was a necessary cofactor for phosphorylcholine incorporation, while the inference was that choline was not influenced by this compound, but no data were supplied. In their system the intermediate formed appeared to be a cytidine diphosphatecholine which could then react with an acceptor, presumably a diglyceride, to yield lecithin. When the synthetic derivative was used in the test system, KENNEDY and WEISS[42] found a much higher rate of incorporation than with phosphorylcholine alone. Thus, it would appear from these results, that phosphorylcholine represents an important precursor of lecithin, and choline could be incorporated by prior conversion to phosphorylcholine, most probably by the system described by WITTENBERG and KORNBERG.[73]

From these investigations, it would appear probable that all the enzymes responsible for the synthesis of lecithins are localized within one particular tissue. In addition, it appears certain that the entire sequence of events occur in a stepwise manner. Whether multiple pathways are involved in this synthetic scheme, or whether the above proposals can be assimilated into a single route, remains to be clarified. It is well to point out that, though not mentioned here, there is the strong possibility that a dehydrogenase system(s) may be important in this reaction scheme. Finally it is evident that the precursors may be derived from other lipides, i.e. diglyceride from the triglycerides, and also from carbohydrates, i.e. glycerophosphoric acid from the glycolytic intermediates such as dihydroxyacetone phosphate.

D. Oxidation-reduction systems

A particularly intriguing question in the area of lipide biochemistry is the possible participation of the phospholipides in oxidation-reduction reactions and the enzymes which are effective in these transformations. The most obvious site of oxidative attack on the lecithin molecule would be the fatty-acid units and as a consequence the most attention has been directed toward this possibility. It is as yet not clearly defined as to the chemical form in which long chain fatty acids may be oxidized; whether as the "free acylcoenzyme A" unit, a glyceride,

or a phospholipide. It is a well known fact that $\Delta 9:10$ fatty acids can be synthesized in mammalian tissues, but the enzyme systems and the "typical" substrates for such reactions have not been identified. In general, two major areas of investigation have been explored; one, involving the possible role of lecithins in the oxidation-reduction systems in the mitochondria; two, concerning the actual dehydrogenases capable of attacking these lipides.

1. *Phospholipide involvement in mitochondrial reactions*

As is well established, the mitochondria of mammalian cells contain a high concentration of lipides, of which the major component is phospholipide. One school of thought has considered the possibility of the phospholipides functioning mainly as a "cementing" substance for the various mitochondrial enzymes or possibly spacing the enzymes at the appropriate distance and site, and the second school of thought tends to believe that the phospholipides may function as electron carriers.

Kielley and Meyerhof[43] studied the possible relation of lecithin to the activity of Mg-ATPase of muscle. When *Cl. welchii* toxin was allowed to act on this enzyme, they noted a linear correlation between the amount of acid-soluble phosphorus released and the degree of ATPase inhibition. No inhibition was noted when the products of the reaction were added to fresh ATPase preparations. When calcium ion was omitted, no inhibition by the toxin was observed. They considered that a phospholipide component of partially purified Mg-ATPase of muscle is essential for activity of this enzyme.

When heated snake venoms were added to various enzyme systems, Braganca and Quastel[8] reported an inhibition of enzymes such as succinoxidase, which is associated with the mitochondria of the cell. Soluble enzymes such as those involved in the glycolytic scheme are not attacked. They concluded that the lecithinases in the venoms caused degradation of mitochondrial structures upon whose integrity or particular spatial configuration the enzymes depend for optimal activity. In a study of the effect of lecithinase *A* of snake venoms or pancreas on oxidative enzymes, Nygaard and Summer[59] found succinoxidase of rat liver to be completely destroyed. No reversibility of the reaction could be demonstrated. When fresh mitochondria were treated with varying amounts of incubated lecithinase *A* treated mitochondria, no loss in activity except that due to residual lecithinase *A* could be noted. Though not clearly defined, they reasoned that lytic action was not involved. They concluded that lecithin was the specific target for the venom action and that perhaps a lecithin-containing compound may be located between succinic dehydrogenase and cytochrome *c* wherein a specific function possibly in oxidation-reduction or some spatial requirement, must be maintained.

Edwards and Ball[20] investigated the inactivating effect of *Cl. welchii* toxins and cobra venom on the succinate complex of enzymes. When the toxin was used, they reported a concomitant increase in acid soluble phosphorus with a decrease in the succinoxidase activity. Inactivation of succinoxidase never exceeded 70 per cent, while succinate dehydrogenase and cyto-

chrome oxidase activities were inhibited to even a lesser degree. Snake venom at very low concentrations could completely inactivate succinoxidase, while the other two enzymes were only partially inhibited. In the absence of calcium, both toxin and venom were ineffective. Boiled venom retained its inactivating effect. When sodium oleate was added to the systems a strong inactivation similar to that of the venom was noted. Sodium stearate was much less effective as an inhibitor. They concluded from these results that the phospholipides were important to succinoxidase activity, but did not define their role. However, they did rule out, on the basis of the diffuse action of the toxin rather than a localized attack, any coenzyme function for phospholipide.

Although it would appear highly probable from these studies that phospholipide is involved in some nonspecific function in the oxidation-reduction systems in the mitochondria, certain other conclusions can also be reached. In most of the above studies, the lecithinase sources used cannot be truly regarded as containing a single type of enzyme. Hence the breakdown of lecithin may only be a secondary reaction whereas the main attack may be made by another enzyme, perhaps a proteolytic one, which may be the more specific. In addition, requirements for calcium ions (or metal ions in general) and heat stability are not necessarily a definition for the lecithinases.

2. *Evidence for lecithin dehydrogenases*

In an interesting study, Bernheim and Bernheim[7] noted the influence of vanadium on the oxidation of phospholipides by certain tissues. When crude phospholipide preparations were added to thoroughly washed liver suspensions containing vanadium (as vanadate), a rapid uptake of oxygen occurred. In the absence of the metal the uptake was significantly lower. Vanadate alone or phospholipides alone caused very little net oxygen uptake. Although direct proof was not secured, they concluded that the oxidation occurred in the fatty acid portion of the molecule. Fatty acids plus vanadate did not cause any significantly rapid uptake of oxygen. On the basis of iodine numbers of crude fractions, they concluded that an increase in the double bonds had occurred. Elliot and Libet[21] found that the oxidation of phospholipides by rat brain or liver suspensions was catalysed by ascorbic acid and in addition by the presence of small amounts of an iron complex called ferrin, from liver. Of the brain lipides used as substrates, only cephalin underwent oxidation. Linoleic and linolenic acids were oxidized but at a much slower rate than mixed phospholipides or cephalins.

Annau *et al.*[2] isolated an enzyme from beef liver, which could in the presence of hypoxanthine or xanthine dehydrogenate lecithin (and also fatty acids). The enzyme was not identical with xanthine oxidase. Although no evidence was supplied on the nature of the lecithins used or the products, it was evident that oxidation had occurred and that this required the presence of hypoxanthine or xanthine. Of interest was the fact that only saturated fatty acids could be attacked by this system, whereas unsaturated acids were not.

With crude beef brain phospholipides or beef lung hydrolecithin as substrates,

O'Connell and Stotz[60] have demonstrated an apparent ATP dependent oxidation of these compounds in whole homogenates of rat liver. Unlike free fatty acids which gave rise to acetoacetate under these conditions, these phospholipides did not. In the presence of ATP plus phospholipide in a liver homogenate, reduction of methylene blue was noted in a typical Thunberg reaction. In the aerobic reaction, however, there was no increase in the iodine number of the phospholipide; no evidence was supplied as to the means by which the phospholipides were isolated or assayed. On the basis of the acetoacetate formed only from free fatty acids and none from the intact lipide, they considered no release of fatty acids from the phospholipide during oxidation. In this system, it would be of interest to know whether any other portion of the molecule had been attacked or whether the phospholipide was catalysing the oxidation of some other component of the homogenates.

Although these investigations have indicated the possible presence of lecithin dehydrogenases in tissues, it would be desirable to investigate these systems in more detail with highly purified substrates and enzymes. In addition, isolation and identification of the end products of these reactions would aid considerably in an understanding of the mechanism of action of these enzymes.

IV. Summary

It appears certain now that there are at least four well established hydrolytic enzymes which can attack lecithin and lysolecithin, namely lecithinase *A*, *C*, and *D*, and lysolecithinase *B*. Although each enzyme is distinctly different in its action, the proof of the exact individuality of each system shall have to await further purification studies. A brief summary of the characteristics of each of these enzymes is presented in Table 5.

Table 5

Enzyme	*Typical source*	*Usual substrate*	*Products of reaction*	*Other substrates*	*Remarks on reactions*
Lecithinase *A*	Snake venom	Lecithin*	Lysolecithin and free fatty acid	Phosphatidyl ethanol-amine (slow)	Active in di-ethyl ether or water
Lysoleci-thinase *B*	*P. notatum*	Lysolecithin*	GPC and free fatty acid	Unknown	Not activated by ether
Lecithinase *C*	Carrot, cabbage	Lecithin	Phosphatidic acid and free choline	Phosphatidyl ethanol-amine	Activated by ether
Lecithinase *D*	*Cl. welchii* toxin	Lecithin*	Diglyceride and phos-phoryl choline	Sphingo-myelins (slow)	Active in ether or water

* Saturated or unsaturated.

As has been emphasized here, these enzymes can be very useful tools in the proof of structure of particularly labile or difficultly degradable compounds,

and in providing derivatives which are at present not obtainable by any other route. Moreover, the employment of these enzymes in the interpretation of the possible metabolic routes of the phospholipides would appear to be most promising. Finally the improved techniques available for the purification of enzymes allows the possibility of the isolation of homogeneous, individual lecithinases.

As would be expected, it is highly probable that the enzymes involved in the synthesis of lecithin are completely different than those concerned with the hydrolytic pathway. Future study on this subject will undoubtedly centre about the possible intermediates en route to lecithin and the purification of the individual enzymes responsible for these conversions. The existence of specific lecithin dehydrogenases has not been definitely established and further investigation on this topic would appear warranted.

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LIPID DYNAMICS IN ADIPOSE TISSUE

B. Shapiro

CONTENTS

		Page
I.	INTRODUCTION	178
II.	SOME FACTORS DETERMINING THE COMPOSITION OF ADIPOSE TISSUE	178
	A. Effect of the diet	179
	B. Effect of location and temperature	180
III.	FATTY-ACID SYNTHESIS	181
IV.	MOBILIZATION AND DEPOSITION OF DEPOT FAT	186
	A. Methods of investigation	186
	B. Humoral regulation	187
	1. Pituitary gland	187
	2. Adrenal glands	188
	C. Nervous regulation	189
	D. *In vitro* systems	189
	1. Fat uptake by adipose tissue	189
	2. Release of fatty acids	193
	REFERENCES	194

7

LIPID DYNAMICS IN ADIPOSE TISSUE

B. Shapiro

I. Introduction

The interest in the physiology and biochemistry of adipose tissue has increased considerably during the last few years. This is mainly due to the indications which became available that lipid mobilization from this tissue and the deposition of fat in the tissue may play an important role in the etiology of obesity, in diabetes acidosis, in various lipaemias, and in atherosclerosis. The conviction, now generally accepted, that adipose tissue participates actively in lipid metabolism and especially in lipid transport, calls for a better understanding of the biochemical activity of this tissue. With the aid of this knowledge it may be possible to shed some light on the factors and conditions which operate in normal lipid homeostasis and in deviations from normality.

Considerable evidence has accumulated to show that the fat cell can execute most metabolic acts found in other cells, but that enzymes of adipose-tissue, concerned with lipid metabolism, are especially active (Shapiro and Wertheimer, 1956). This is in line with the adaptation of this tissue to fat metabolism, which enables it to perform its specific physiological function. However, very little is known about the function of these enzymes in the regulation of fat formation, deposition, and mobilization, or the dependence of their activity upon various physiological or pathological factors which induce changes in lipid homeostasis. In the present paper, an effort will be made to define some of these problems and to summarize experimental work related to their solution. No attempt has been made to cover the entire literature on adipose tissue and the reader is referred to some recent general reviews in this field (Clement, 1950; Eger, 1954; Polonovski and Polonovski, 1953; Shapiro and Wertheimer, 1956; Wertheimer and Shapiro, 1948; Wells, 1940).

II. Some Factors Determining the Composition of Adipose Tissue

Adipose tissue represents a considerable part of the whole body. It constitutes around 18 per cent of the body weight of the normally fed human (Wells, 1940) and about 13 per cent in the rat (Reed, Anderson, and Mendel, 1930). The water content, generally, does not exceed 30 per cent. Of the dry matter about 5 per cent is represented by proteins and the bulk (90 per cent and more) by lipids. Most of the lipids in adipose tissue are triglycerides (more than 99

per cent). Cholesterol (0·24 per cent) and phospholipids are present in very small amounts.

The specific composition of animal body fat has been treated extensively by HILDITCH (1947) and the factors determining the fatty-acid composition of depot fat have been amply reviewed by SHORLAND (1955). It is not necessary, therefore, to deal with these aspects in the present review, and only several points, pertinent to the activity of adipose tissue, will be elaborated.

A. Effect of the diet

It has been shown repeatedly that the fat stored by each species is a comparatively constant mixture of glycerides (HILDITCH, 1947), differing from that deposited in other species in the proportions of individual fatty acids. This specific character can be greatly modified by the ingestion of large quantities of a particular fatty acid. As has been shown by SCHOENHEIMER and RITTENBERG (1935) and by BERNHARD and STEINHAUSER (1944), fat absorbed from the intestine is deposited in adipose tissue to a large extent, irrespective of the nutritional adequacy of the diet. It is thus easy to understand that the composition of the dietary fats markedly influences that of the depots.

When rats were fasted and subsequently fed a diet containing a high percentage of corn oil, their depot fat was found to be almost identical in its fatty-acid composition with that of corn oil (LONGENECKER, 1939b), being especially rich in highly unsaturated fatty acids. Upon utilization of this depot during inanition only slight changes in the molecular proportions of the component fatty acids was produced, indicating that mechanisms for the interconversion of the various fatty acids, though present (see later), are limited in their capacity. On the other hand, when an oil of low iodine value such as coconut oil (Iodine value (IV = 8) was ingested, the fatty acids deposited were more unsaturated than those fed, having an iodine value of 31 (LONGENECKER, 1939c). During inanition the degree of unsaturation increased still further. These findings point to a higher capacity of adipose tissue to deal with saturated fatty acids, either by desaturation or by preferential catabolism or mobilization. When diets were fed, which did not cause such large-scale deviations from the specific composition of the animals depot fat, no evidence for selectivity in mobilization of fatty-acid components during starvation was obtained (HILDITCH and PEDELTY, 1943).

Upon feeding the rats with high-carbohydrate or high-protein diets, the fat stored was found to be especially rich in C_{16} acids, saturated and unsaturated (LONGENECKER, 1939a), pointing to the predominance of these acids when the main source of fat is endogenous synthesis. In this case, too, no change in composition of the fatty acids was found during utilization.

Most evidence shows that there is a tendency of depot fat to return to its specific composition following a forced dietary change. The rapidity with which this return is established seems to depend upon the gross composition of the fat in the diet as well as on other factors, such as essential fatty acids (NORCIA

and LUNDBERG, 1954) and other undefined factors (TOVE, SMITH, YOUNG, and SHERWOOD, 1954).

The establishment of the specific fatty-acid mixture can be brought about by selective deposition or mobilization, endogenous fat formation and decomposition, as well as by interconversion of the depot fatty acids. This latter capacity has been demonstrated by SCHOENHEIMER and RITTENBERG (1937) with the aid of deuterated fatty acids. When deuterostearic acid was ingested, deuteropalmitic acid and deutero-oleic acid were found in the depot fat. Similar conversions of 1-C^{14}-myristic acid to myristoleic acid, oleic acid, and palmitic acid have been shown by ANKER (1952).

The animal body is thus capable of carrying out desaturation as well as shortening and lengthening of the fatty-acid chain. Since adipose tissue has been shown to contain a desaturating enzyme (QUAGLIARIELLO, 1932; YOSII, 1937; SHAPIRO and WERTHEIMER, 1943; CHAMPOUGNY and LE BRETON, 1947), it seems likely that some of these transformations of fatty acids are carried out in the depot itself. In the light of this observation it is doubtful whether the constancy of the fatty-acid composition during inanition, mentioned before, is proof of a nonselective mobilization of fatty acids. The fatty-acid desaturase may renew a certain equilibrium mixture of fatty acids when the composition is temporarily changed by selective mobilization or deposition.

Recently, acetal phosphatides were found to be present in adipose tissue of the newborn rat (YARBORO and ANDERSON, 1956). These substances rapidly disappear concommitantly with the appearance of fat in the tissues. It was postulated that they are involved in some manner in the laying down of fat in adipose tissue.

B. Effect of location and temperature

The location of the tissue has also been found to effect marked differences in fatty-acid composition. HENRIQUES and HANSEN (1901) were the first to compare the composition of fat stored under the skin and within the abdominal cavity. In the dog, rabbit, and duck there was little difference in iodine number. In the horse, sheep, camel, or goose there was a greater difference; and in the hog or ox a very great difference. Fat in the superficial layers of adipose tissue has been found to be less saturated and to have a lower melting point than fat in depots more deeply situated in the body. This has been explained teleologically by a presumed tendency to regulate fat composition so that it remains fluid at the temperature which prevails in each part (HILDITCH, 1947). Starting from this assumption, FAWCETT and LYMAN (1954) studied the effect of low environmental temperature upon the iodine value and solidification point of body fat in hibernating animals, the hamster and the ground squirrel. These animals will tolerate a fall in body temperature of 30°C without ill effects (LYMAN, 1948). Since their respiratory quotient is about 0·7, fat is presumably the main fuel throughout the dormant period. One would therefore expect that the fat remains fluid even at only a few degrees above 0°C. It was found that fat of hamsters which had been kept in the cold room had an average iodine

value of 87·3 compared with 83·7 for animals kept in a warm room. In rats, no effect was found on the saturation of fat upon exposure to cold (see however CLEMENT, 1949).

This finding suggested that the lowering of the melting point of depot fat may be a necessary preparation for hibernation, and that the delay from first exposure to cold to the onset of hibernation was related to the time required to affect the necessary change in the composition of depot fat. However, changing this composition by dietary means had no influence on the latent period.

A specific role in hibernation has been claimed for brown adipose tissue. Thus, HOOK and BARRON (1941) found a low-temperature coefficient for the respiratory activity of this tissue, so that a considerable activity is maintained even at low temperature.

Although the effect of temperature on the iodine number of fat deposits seems to be established in many cases, the considerable differences in reactivity found with various species points to factors other than local temperature which may account for variations in saturation of various fat depots.

Here too the fatty-acid desaturase of the tissue may play a decisive role. The equilibrium mixture of fatty acids established by this enzyme system may be changed by different temperatures. It remains also to be shown whether the species differences mentioned may not be due to differences in activity of this enzyme.

III. Fatty-acid Synthesis

Conclusive evidence has accumulated during the last ten years to show that adipose tissue is capable of transforming carbohydrates into fat, and that a considerable part of the endogenous fat stored is produced in the adipose-tissue cells. A rapid gain in endogenous depot fat can be demonstrated with the following dietary regimes. (*a*) When animals are undernourished or starved and then fed a carbohydrate-rich diet (TUERKISCHER and WERTHEIMER, 1942); (*b*) when forced to ingest excessive quantities of carbohydrates (TUERKISCHER and WERTHEIMER, 1942); (*c*) after injection of appropriate doses of insulin (HAUSBERGER and GUJOT, 1937b); (*d*) in rats trained to consume their daily food ration in 1 hr (TEPPERMAN, BROBECK, and LONG, 1943); (*e*) in rats starved and fed on alternate days (MACKAY and DRURY, 1941). In all these regimes, increased fat deposition has been found to be preceded by deposition of glycogen (WERTHEIMER, 1945). This deposition is transient and glycogen disappears concurrently with the beginning of measurable fat accumulation. No glycogen can be detected in adipose tissue of normally fed rats and dogs. Glycogen deposition thus seems to be an indicator of increased synthetic activity in these tissues. Another interesting case is embryonic adipose tissue which contains glycogen but only negligible amounts of fat. Immediately after birth the glycogen content decreases and disappears and fat starts to accumulate (HAUSBERGER and GUJOT, 1937a).

The glycogen deposited is located inside the cells, and in view of its large

molecular size one would expect that it was newly formed in the tissue rather than preformed glycogen carried by the blood. This assumption has been substantiated by experiments, showing that adipose tissue, *in vitro*, incorporates radioglucose into glycogen (Rose, Stern, and Shapiro, 1953; Rose and Shapiro, 1955). In some conditions also a net synthesis of glycogen could be demonstrated *in vitro* (Hausberger and Jachtorowisz, 1949). The problem arises whether glycogen is the precursor of the fatty acids, as suggested by the correlation and time sequence of their appearance (Tuerkischer and Wertheimer, 1942), or is a side product of fat synthesis from glucose, which accumulates in the cells when this process is especially rapid. (For another explanation, see later.) It has been shown that adipose-tissue glycogen has no glucogenic function similar to that of liver glycogen, since it is not degraded to glucose (Mirski, 1942).

Concurrently with the appearance of glycogen, the tissue exhibits an elevated respiratory quotient, exceeding 1 (Ruska and Oestreicher, 1936; and Mirski, 1942). Also, when glucose, lactate, acetate, pyruvate, or succinate are added to adipose tissue of rats starved for 24 hr, the respiratory quotient rises to figures exceeding those expected if the added substrates were completely burned (Haugaard and Marsh, 1952). Conclusive evidence for the capability of adipose tissue to synthesize fatty acids was brought by Shapiro and Wertheimer (1948). It was shown that deuterium was introduced into the fatty acids of adipose tissue incubated in serum enriched with deuterium oxide. Due to the large quantities of fat present in the tissue, the concentration of deuterium containing fatty acids (i.e. newly formed fat) was low, but still exceeded the limits of detection by the falling-drop method (Keston, Rittenberg, and Schoenheimer, 1937) five to ten times.

Higher deuterium concentrations were found with tissues of rats in conditions of accelerated fat deposition, i.e. after a period of undernutrition and realimentation. Interscapulary brown fat was more active than mesenteric fat, while liver was two to six times more active than adipose tissues in exchanging its fat. This relationship between the exchange rates of fat in liver and adipose tissue is similar to that found *in vivo* (Schoenheimer and Rittenberg, 1935; Stetten and Grail, 1943; Pihl, Bloch, and Anker, 1950), i.e. half life times for liver fatty acids between 1·5–3 days and for depot fatty acids between 5–20 days, depending on nutritional conditions. If a large part of adipose fat were derived from the liver, one would expect that the ratio of exchange rates in these two tissues would increase in favour of the liver when the isolated tissues are tested. Since this was not the case, the results signify that the bulk of newly formed fat deposited in adipose tissue is formed in the tissue itself, and that the total amount of fat formed in adipose tissue exceeds that formed in liver, in view of the much larger quantities of fat in the former.

These earlier results with deuterium oxide were later confirmed with C^{14}-labelled precursors of fatty acids. Radioacetate, radioglucose, and radiopyruvate (Rose, Stern, and Shapiro, 1953; Feller, 1954; Hausberger, Milstein, and Rutman, 1954; Favarger and Gerlach, 1954; Rose and Shapiro, 1955)

were shown to be incorporated into adipose tissue fatty acids *in vitro*. Degradation of the fatty acids synthesized from acetate and glucose (ROSE *et al.*, 1953) showed that these precursors participated in a complete synthesis of the fatty-acid molecule and not merely in the addition of two carbon atoms to an existing fatty-acid chain.

FELLER (1954), who demonstrated the conversion of radioacetate into fatty acids by slices of inguinal subcutaneous fat, reported the puzzling finding that the carboxyl carbon of acetate was converted to fatty-acid carbon at a rate twice that of the methyl carbon. CO_2 formed from acetate was shown not to be an intermediate in fatty-acid synthesis, since C^{14}-bicarbonate was not converted to fatty acids. Comparing the rate of synthesis in adipose tissue with that in liver, FELLER came to the conclusion that in the presence of glucose and succinate as cosubstrates, adipose tissue formed fatty acids at a rate exceeding that in liver, when values were referred to the fat-free tissue or to the total nitrogen content.

On the other hand, only small amounts of cholesterol or other nonsaponifiable lipids were formed, although appreciable amounts were found in the tissue. Cholesterol seems to be derived mainly from that carried to the tissue by the blood.

With radioglucose as substrate, HAUSBERGER *et al.* (1954) compared the amount of glucose converted to fatty acids to that oxidized to CO_2 for liver and adipose tissue. It was found that a much greater percentage of carbons and energy derived from glucose was channelled into fatty-acid formation in adipose tissue than in liver. This points to the importance of adipose tissue in fat metabolism. Insulin injection was found to enhance fat formation in both tissues, when tested *in vitro*, while no measurable activity was discovered in tissues obtained from alloxan diabetic rats. The rates of incorporation into fatty acids in adipose tissue exceeded that in liver even when expressed in terms of incorporation per weight of fresh tissue. However, the activity found with liver was exceedingly low, as compared with that obtained by other investigators (CHERNICK and CHAIKOFF, 1950). This is presumably due to the presence of fat in the diet used by HAUSBERGER *et al.* (1954) which depresses fatty-acid synthesis in liver much more than it does in adipose tissue (HAUSBERGER and MILSTEIN, 1955).

Comparison of liver and adipose tissue, with the aid of radioactive precursors, is made difficult by the fact that each tissue requires specific substrates and conditions for optimal activity. Although fatty-acid synthesis in liver is much more rapid with acetate as precursor than with glucose (MASORO *et al.*, 1950), adipose tissue prefers glucose to acetate. Pyruvate exceeds both these substrates as fatty-acid precursor in adipose tissue (ROSE and SHAPIRO, 1955).

The evaluation of the comparative role played by various tissues in fatty-acid synthesis in the intact animal performed by FAVARGER and GERLACH (1955) is therefore highly pertinent. 1-C^{14}-acetate or uniformly labelled glucose was injected intravenously into mice during a realimentation period. Three minutes after the injection, the specific activities in brown interscapular fat

tissue, in mesenteric fat, in the intestine and lungs, were higher than in the liver. At the same time, the blood was practically devoid of newly formed fatty acids, making transport from organ to organ highly improbable. They came to the conclusion that the role of the liver in the synthesis of depot fat is negligible. When glucose was given *per os*, the difference in specific activity

Table 1. Synthesis of fatty acids in various tissues of the mouse after intravenous injection of radioactive acetate or glucose

From Favarger and Gerlach (1954)

Mouse number	28	29	30	31	33	37	39
Weight, gm	25	25	30	18	16	21	17
Weight of total fatty acids, mg	1307	1223	1400	835	1264	2790	670
Total activity of fatty acids in 10^3 counts/min	106	205	119	127	63	238	352
Total activity of fatty acids in the liver in 10^3 counts/min/mg	0·4	0·8	0·85	0·65	2·4	2·9	3·3
Specific activity of liver fatty acids in counts/min/mg	32	53	33	29	23	15	114
Concentration of fatty acids in the liver in %	1·8	1·9	3·1	3·0	13·3	19·8	6·7
Specific activity of fat in the omentum in counts/min/mg	18	192	63	250	22	84	1740
Specific activity of fatty acids in brown fat in counts/min/mg	169	490	319	800	405	455	—
Concentration of fatty acids in the omentum in %	53·2	53·2	23·4	14·5	12·0	57	4·0
Specific activity of fatty acids in the lungs in counts/min/mg	—	—	1395	3390	262	472	1000

Mice No. 28–31 received $2{\cdot}7 \times 10^6$ counts/min of $CH_3{}^{14}COONa$ and were killed after 3 min. Mice No. 33, 37, 39 received $11{\cdot}7 \times 10^6$ counts/min of radioglucose and were killed after 12 min. All the animals were fasted for 48 hr and fed 0·7–1·0 gm of bread 30 min before the injection.

between adipose tissue and liver still persisted, although it was smaller than when given intravenously. Adipose tissue of the rat was found to be less active than that of the mouse. The relative activity of liver fatty acids gradually rises when longer-term experiments are conducted, and after several hours the specific activity of fatty acids in the liver exceeds that of adipose tissue markedly, as shown in earlier experiments of Stetten and Grail (1943) and Pihl, Bloch, and Anker (1950). This discrepancy between short- and long-term experiments might be explained by the much higher dilution of the first radioactive precursor reaching the liver with the endogenous acetate pool than is the case in adipose tissue. Furthermore, Zabin (1951) has shown that in experiments of short duration (up to 6 hr) with radioacetate, the specific activity of the carboxyl carbon of stearic acid was fifteen times that of the average chain carbon. Thus, a considerable part of the incorporation of radioacetate in this type of experiment is due to the elongation of existing carbon chains.

However, whatever the case may be, the experiments of FAVARGER and GERLACH (1955) show without doubt that fat formation in adipose tissue can proceed independently of the liver.

Another approach for studying fat synthesis by adipose tissue was suggested by CLEMENT and CHIMENES (1954), who injected insulin into inguinal adipose tissue on one side and compared the composition of the lipids in the injected site with its symmetrical counterpart. Eighteen hours after the injection an increase in tissue weight and fat content was found in the injected site. At the same time, the iodine value of the fatty acids in the injected tissue fell, as compared with the noninjected side. Since it is known that newly synthesized fatty acids are predominantly saturated, this finding would suggest that insulin exerted a local acceleration of fat synthesis. Although this interpretation is the most likely, the authors drew attention to the possibility that the increase in degree of saturation might be due to preferential mobilization of unsaturated fatty acids.

Experiments with radioactive precursors again confirmed that the nutritional state of the animal, from which the tissues were obtained, has a marked influence on the rate of synthesis. Dietary regimes, which enhance fat accumulation *in vivo*, also reveal higher rates of incorporation *in vitro* (ROSE, STERN, and SHAPIRO, 1953; FAVARGER and GERLACH, 1954; ROSE and SHAPIRO, 1955). Similar to findings with liver (MASORO *et al.*, 1950), prolonged starvation caused a marked decrease in fat synthesis. This decreased synthesis was overcome by a short period of refeeding, after which the fat synthesis was at its maximum, exceeding that of tissue in continuously fed animals. This depression in fat synthesis in starved animals was found to about the same degree, whether acetate, glucose, or pyruvate-2-C^{14} served as precursor (ROSE and SHAPIRO, 1955). Concurrently with the depression in fat synthesis, the formation of $C^{14}O_2$ from radioglucose was also inhibited by 90 per cent. With 2-C^{14}-pyruvate and 1-C^{14}-acetate no depression in $C^{14}O_2$ was found in tissues of starved animals as compared with those of refed ones. These results point to a conclusion similar to that advanced by HIRSCH, BARUCH, and CHAIKOFF (1954), in their work on fat synthesis in mammary tissue. A certain type and rate of glucose metabolism seems to be necessary in order to induce fatty-acid synthesis from its precursors. Pyruvate and acetate, although their utilization and oxidation to CO_2 is not interrupted, do not give rise to fatty acids when glucose degradation is interrupted. Addition of glucose partly removes the block in fatty-acid synthesis from acetate.

The site of inhibition in starved adipose tissue is not located at the hexokinase step, since the overall utilization of glucose is inhibited by only 30 per cent while CO_2 and fatty-acid production continues at the rate of a small fraction of that in refed tissues. At the same time the conversion of glucose into glycogen is not inhibited, but perhaps rather enhanced. (The contradiction of this finding with that reported previously by HAUSBERGER and JACHTOROWISZ (1949), who found a depression in net glycogen synthesis in starved tissues as compared with that in refed ones is, so far, difficult to reconcile.) This would place the blocked

reaction beyond the hexosephosphate step or to a changed pathway of glucose utilization during starvation.

These findings may also serve as an explanation of the previously mentioned fact that glycogen accumulates in adipose tissue only following a period of undernutrition or starvation, followed by realimentation, and is absent in tissues of continuously fed animals. Starvation introduces a block in glucose dissimilation to CO_2 and fatty acids but leaves the pathway to glycogen open. In the presence of this block, when glucose supply is renewed by alimentary glucose or by insulin injection, glycogen can accumulate. When feeding is continued, the block is gradually removed and glycogen is converted to fatty acids and CO_2.

IV. Mobilization and Deposition of Depot Fat

Mobilization and deposition of fat in adipose tissue is a continuous dynamic process, as shown in the classical work of Schoenheimer and Rittenberg (1936) and is one of the major processes in the energy metabolism of the animal organism. The bulk of food fat (Sinclair, 1935) as well as a considerable portion of the carbohydrates ingested (Stetten and Boxer, 1944) is converted into adipose-tissue fat. On the other hand, during dietary inadequacy the fat depots represent the main caloric reserve of the organism.

The mechanism whereby the fat depots are activated to release or accumulate fat are still very little understood. A homeostatic mechanism, by which a decreased fat concentration in the blood brings about an increase in fat mobilization, does not seem to exist. Enhanced fat mobilization during inanition proceeds generally in spite of an increased fat content of the blood (Bloor, 1943).

The active metabolism of adipose tissues seems to be an important factor in the dynamics of depot lipids, as is suggested by the conclusive evidence for the participation of nervous and humoral impulses in the acceleration or retardation of fat mobilization. At some stage, a direct interaction must exist between these factors and the adipose cells and enzyme systems.

A. Methods of investigation

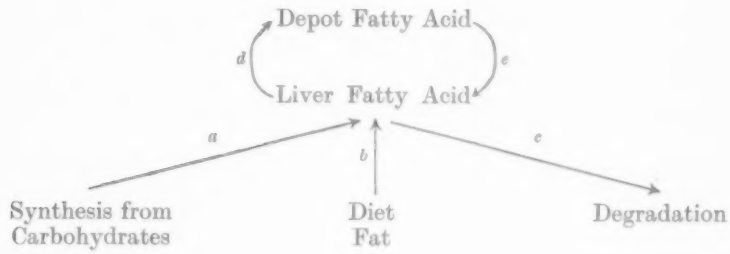
The methods for studying fat mobilization from the depots are based either directly on the determination of increases or decreases in carcass fat, or indirectly on the production of lipaemia or on the increase in liver fat.

Accumulation of fat in the liver of the mouse was found to be an especially sensitive and convenient demonstration for enhanced fat mobilization from adipose tissue. In the case of the indirect methods, evidence has to be provided that the increased fat content of blood and liver derive from depot fat.

The scheme of Stetten and Salcedo (1945) given on facing page illustrates the possible precursors of liver fat.

Liver fatty acids may be derived from one of the sources *a*, *b*, or *e*. When deuterium oxide is given to the animal, newly formed fatty acids will contain

deuterium. Due to the more rapid exchange of liver fatty acids than that of the depot fatty acids, liver fatty acids will contain a higher deuterium concentration than those in the depot. Any factor, which now accelerates pathway *e*, i.e. mobilization of depot fat to the liver, will bring fatty acids of lower deuterium content to the liver and will decrease the deuterium concentration in the liver fatty acids. Liver fat derived from any of the other sources will have quite different effects. Pathway *a* will increase the deuterium concentration in liver fat, since fatty acids newly formed in the presence of high D_2O concentration in the body water will also contain a high deuterium concentration. Pathway *b* can be excluded if the animal is fasted during the experiment. Pathways *c* and *d* have no influence on the deuterium concentration.



By an earlier method of Barrett, Best, and Ridout (1938), pathway *e* was shown to be accelerated, by feeding the animal deuterated fatty acids, which accumulate primarily in the depot fat, but also bring about higher specific activities in the liver. Any marked shift of fat from the depots will be detected by a decrease in deuterium concentration in the liver fatty acids, which does not, however, fall below that in the depot fatty acids.

B. Humoral regulation

1. *Pituitary gland.* Best and Campbell (1936) showed that three days following an injection of an extract of the anterior pituitary gland into normal rats, there was a reduction in their depot fat and an increase in liver fat. The source of the fat deposited in the liver was shown to be the depot fat. The reduction in carcass lipids was found to involve almost exclusively the glycerides (Campbell and Lucas, 1951). There was no reduction in cholesterol or cholesterol esters and only a negligible change was found in the phospholipids. The rate of mobilization of fat from adipose tissue under the influence of the pituitary factor was dependent on the glyceride content of the tissue.

The pituitary is implicated as the agent which is also responsible for increased fat mobilization during fasting. Liver fat was found not to be increased in the hypophysectomized animal as a response to fasting as it is in normal animals (Levin and Farber, 1952). Weil and Stetten (1947) found that urine of fasted rabbits contains a factor which causes fat accumulation in the liver, when injected into mice. No such factor was found in the urine of fed rabbits. The

factor, named "adipokinin", could be concentrated by alcohol precipitation, and was extracted from the precipitate by means similar to those used for extraction of the active principle of the hypophysis. However, no direct proof for its origin from the pituitary was obtained.

According to LEVIN and FARBER (1952) the "fat-mobilizing hormone" is not identical with ACTH, as neither ACTH nor cortisone alone were able to induce fat transfer to the liver. On the other hand, the pituitary factor was inactive in the adrenalectomized animal. When pituitary extract was given together with cortisone, fat mobilization was renewed in adrenalectomized animals.

It was concluded that ACTH in the normal animal or cortisone makes the animal more responsive to the "fat-metabolizing hormone." Some preparations of growth hormone can replace the pituitary factor, but it is still questionable whether growth hormone and fat-mobilizing activity reside in identical pituitary fractions. PAYNE (1949) studied the fat-mobilizing activity of crude pituitary fractions and was unable to find any correlation between this activity and any of the other defined activities. ASTWOOD, RABEN, ROSENBERG, and WESTERMEYER (1953), and ROSENBERG (1953) purified ACTH from growth hormone and other trophic hormones. Three micrograms of this preparation caused considerable mobilization of depot fat in fasted mice. However, this activity was not due to a corticotrophic action, since it was also present in adrenalectomized and hypophysectomized mice maintained with cortisone. They came to the conclusion that pituitary "adipokinin" is a distinct principle and not identical with other pituitary factors.

Recently SEIFTER and BAEDER (1956) obtained a factor, named "lipid mobilizer" (LM), from plasma of dogs, rats, and horses treated with cortisone. Four hours after the cortisone treatment (5 mg/kg) dialysates were prepared from plasma and freeze-dried. When injected into fasted mice, rats, rabbits, guinea pigs, dogs, and humans, a marked elevation in plasma lipids was obtained. Dialysates from untreated animals were 1/1000 as potent. No LM was produced by cortisone treatment of hypophysectomized animals. The hyperlipaemia persisted for at least one week in rabbits and dogs, when a single dose was given. When dogs received weekly injections of LM, marked depletion of their various adipose tissues was obtained. The LM factor was found not to be identical with cortisone, but was released by the injected cortisone. Although presence of the pituitary was obligatory for this release, the factor was not identical with growth hormone, since it proved to be dialysable and heat stable and had no growth promoting activity.

2. *Adrenal glands.* WOOL and GOLDSTEIN (1953), used ethionine treatment for the production of fatty livers in rats. Ethionine, being an anti-metabolite of methionine, prevents the lipotropic effect of methionine and thus inhibits the rate of fatty acid dissimilation in the liver. In this case even normal amounts of fat mobilized from adipose tissue cause an increased fat content of the liver (about 15 per cent). No fatty livers were produced in adrenalectomized animals by ethionine treatment. The injection of cortisone (in doses ranging from 0·4–10 mg per rat) did not raise liver fat above 10 per cent. However, when

cortisone was given together with epinephrine, fatty livers were produced. Epinephrine alone was inactive in the adrenalectomized animal.

The action of epinephrine on fat mobilization was also shown directly by these authors. Demedullated rats or rats treated with ergotamine (blocking the action of epinephrine) did not develop fatty livers (8·3 per cent fat versus 14·6 per cent in the control) when ethionine was given. With epinephrine, but not with cortisone, liver fat reached values approaching those in unoperated controls (12·5 versus 14·6 per cent.) Here again, cortisone, though inactive by itself, facilitated the mobilization process triggered by epinephrine. In the normal animal, as well, epinephrine injections were shown to lead to a decrease in carcass fat (CULLUMBINE, 1952) and an increase in liver fat (CLEMENT and SHAEFFER, 1937; MACKAY, 1937).

C. Nervous regulation

This action of humoral epinephrine on fat mobilization is in harmony with the action known to be exerted by the autonomous nervous system (SHAPIRO and WERTHEIMER, 1948). Denervation of adipose tissue was shown to result in decreased fat deposition and mobilization. (BEZNAK and HASCH, 1937; HAUSBERGER and GOUJOT, 1937b; and CLEMENT, 1947.) According to KURÉ, THOSHIN, and OKNIAKI (1937) the sympathetic nerves act as inhibitors of fat deposition while the parasympathicus accelerates this process. Normal innervation seems to be requisite for the attainment of a dynamic equilibrium in fat tissue.

D. In vitro systems

1. *Fat uptake by adipose tissue.* In order to arrive at a better understanding of the mechanisms involved, a more simple system is required in which fat deposition and mobilization can be studied. An *in vitro* system which may fulfil some of the requirements was described by SHAPIRO, WEISSMANN, BENTOR, and WERTHEIMER (1948, 1952). When adipose tissue was depleted of the bulk of its fat by prolonged fasting, and then incubated with blood or serum, a decrease in the fat content of the medium was observed. At the same time an increase in fat content of the tissue could be demonstrated by chemical analysis (STERN and SHAPIRO, 1954) as well as by histological observation (STERN, 1953), provided that very little fat remained in the tissue after the starvation period. The contention that the disappearance of fat from the medium consists mainly of transport into the tissue could later be corroborated by using C^{14} labelled fatty acids (SHAPIRO, CHOWERS, and ROSE, 1955) in the incubation medium. This effect is specific for adipose tissue and could not be produced with subcutaneous connective tissue or with the diaphragm.

Uptake of fat from the medium was found to be dependent upon the metabolic activity of the tissue, since heating to 60°C for 5 min, adding NaF M/40 or NaCN M/500, as well as conducting the experiment at 20°C instead of at 38°C, abolished the uptake.

The uptake from serum was confined to neutral glycerides (STERN and

SHAPIRO, 1954). Phospholipids and cholesterolesters did not yield fatty acids to adipose tissue. Similar results were obtained when artificial lipid emulsions were used instead of serum (see Table 2).

Table 2. Uptake by adipose tissue of various fatty acid compounds

From STERN and SHAPIRO (1954)

Compound	*No. of experiments*	*Mean "initial" concentration μ eq/cc*	*Uptake (mean ± standard deviation) μ eq/cc medium*
Triolein	25	15	1·8 ± 0·22
Triolein + M/20 NaF	16	15	1·04 ± 0·27
Lecithin of egg	11	12	− 0·2 ± 0·2
Sorbitan-monolaurate	9	12	4·3 ± 0·44
Sorbitan-monopalmitate	19	14	3·6 ± 0·4
Sorbitan-monopalmitate + M/20 NaF	15	14	1·2 ± 0·35
Sorbitan-monooleate	11	9	2·3 ± 0·35
Sorbitan-monooleate + M/20 NaF	9	9	0·8 ± 0·3
Sodium oleate	9	12	2·4 ± 0·53
Sodium oleate + M/20 NaF	5	12	2·9 ± 0·88

In addition to the neutral glyceride esters, soluble sorbitan esters (Tweens), as well as free fatty acids, were also assimilated. While the uptake of the fatty acid esters was inhibited by NaF, the uptake of free fatty acids was not suscep-

Table 3. Uptake of 1-^{14}C-stearate by adipose tissue

From SHAPIRO, CHOWERS, and ROSE (1956)

	% uptake from medium	*% of fatty acid taken up, found as*	
		$^{14}CO_2$	*ester in tissue*
Refed rats	76	1·4	87
	81	1·1	90
	82	1·3	85
	72	1·4	70
	38	2·4	27
Starved rats	27	16·5	34
	25	4·2	27
	15		8
	51		26
	49		15

tible to this poison. This finding led to the assumption that the ester linkage is attacked during the penetration of fatty acid esters into adipose tissue cells.

When the assimilation of free fatty acids was studied with the aid of 1-^{14}C-stearic acid (STERN and SHAPIRO, 1954; SHAPIRO, CHOWERS and ROSE, 1955, 1956) it was found that a rapid uptake of radioactive carbon took place. This

uptake was much more pronounced with adipose tissue obtained from rats refed for 1–2 days following the starvation period, than with that obtained from starved rats (see Table 3). Tissues from normally fed rats were intermediate in their assimilating capacity for fatty acids. This is contrary to the uptake of esterified fatty acids, which is optimal in starved tissues (SHAPIRO *et al.*, 1952). Upon examination of the tissues, after incubation with C^{14}-stearic acid, it was found that all the C^{14}-carbon removed from the medium could be recovered in the tissue and that a considerable part was present in the esterified fatty-acid fraction. With tissues of starved and subsequently refed rats, 60–90 per cent of the fatty acid was found to be esterified while with tissues of starved rats only 20–50 per cent was found in this form, the bulk being unesterified.

The capacity of the tissue to assimilate fatty acids, is thus correlated to the rate of esterification and in view of the previously mentioned behaviour towards metabolic poisons one may assume that esterification plays a role in fatty-acid uptake. It is doubtful whether the tissue lipase can activate this esterification. On the other hand, SHAPIRO *et al.* (1955) were able to demonstrate the presence of an adenosine-triphosphate (ATP) and coenzyme A (CoA) dependent fatty acid activating system in adipose tissue. This system seems to be similar to the one found by KORNBERG and PRICER (1953) in liver, catalysing the reaction:

$$\text{Fatty acid} + \text{CoA} + \text{ATP} \xrightarrow{Mg^{++}} \text{Fatty acid} - \text{CoA} + \text{AMP}^{*} + \text{P—P}^{\dagger}$$

The fatty acid —CoA complex may now transfer its fatty acid to a glycerol compound and form the ester linkage. Although the exact nature of this glycerol compound has not yet been completely clarified, the formation of glycerides by a CoA and ATP dependent reaction has been demonstrated in liver (TIETZ and SHAPIRO, 1955). It may be pictured as follows:

$$\text{Fatty acid} - \text{CoA} + \text{“glycerol”} \rightarrow \text{glycerol} - \text{fatty acid} + \text{CoA}$$

in which “glycerol” represents the glycerol compound which serves as acceptor. Free glycerol does not serve this purpose, since radioglycerol was not esterified in the above system.

When these results are evaluated for a possible explanation of the mechanism of transport of fat and fatty acids from the blood into adipose tissue cells, one has to keep in mind that this transport does not necessarily require an active process, since the general direction of transfer from a predominantly aqueous medium into the fat droplets of the adipose cell is the one going towards thermodynamic equilibrium. The dependence of fat penetration into the tissue on metabolic integrity points rather to a facilitated transfer, in which the attainment of the equilibrium is accelerated by enzymatic processes. Since the rate of uptake of free fatty acids shows good correlation with the rate of esterification by the tissue, the esterification process seems to be involved in the facilitated transfer of free fatty acids. It may be postulated that the fatty-acid molecule

* AMP = Adenosine monophosphate.
† P—P = Pyrophosphate.

entering the cell boundary becomes bound to the activating enzyme, forming a lipoprotein complex, which can leave the lipid cell boundary and move into the cytoplasm. The fatty acid may now be transferred from the enzyme-CoA complex to an acceptor, to form the fatty-acid ester which is trapped in the fat droplet.

The mechanism of penetration of neutral fat, on the other hand, is so far difficult to understand. It has been shown to be inhibited by fluoride. If one wishes to restrict the assumptions to a single mechanism, one could picture this by assuming a transfer of the fatty acid from the ester to the "fatty-acid activating system" involved in the previously postulated mechanism of transport of free fatty acids (see Fig. 1).

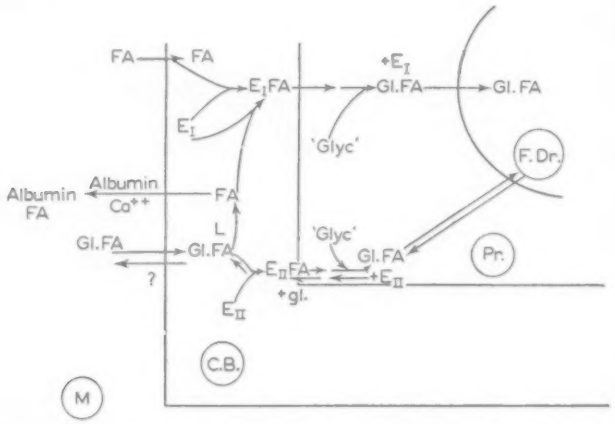


Fig. 1. Hypothetic scheme for the facilitated uptake and release of fatty acids and glycerides in adipose tissue.

M (encircled) = Medium.
F.Dr. (encircled) = Fat droplet.
"glyc" = Glycerol precursor (acceptor of fatty acids).
L = Lipase or lipoprotein lipase.
C.B. (encircled) = Cell border.
FA = Fatty acid.
E_I = Fatty acid activating enzyme system.
Pr. (encircled) = Protoplasma.
Gl. = Glycerol.
E_{II} = Assumed fatty acid transferase.

Alternatively, glycerides might be split by a tissue lipase into free fatty acids which are subsequently assimilated by the mechanism postulated above.

Other mechanism of fatty-acid transport, utilizing phospholipid or cholesterol esters as vehicles, are not corroborated by the results obtained with the tissue *in vitro*, since neither phospholipids nor cholesterol esters yield fatty acids to the tissue. A cholesterol esterase and phospholipase was demonstrated in adipose tissue by SCHOELLY and FAVARGER, (1953). These enzymes were considered to be involved in the fat mobilizing function, since pituitary extracts, which accelerate fat mobilization, also increase their activity. This assumption is not contradicted by the fact that cholesterol esters do not serve as vehicles, since the esterase function may be concerned with intracellular transformations in fatty-acid esters, which facilitate transfer.

We thus have two pathways of fat deposition, one assimilating free fatty acids, and the other dealing with the whole triglyceride molecule. The first pathway shows a high activity, since adipose tissue under suitable conditions can remove up to 90 per cent of the fatty acids from an albumin-fatty acid complex even when the fatty acid concentration is very low (1–4 microequivalents/ml). Uptake of triglycerides is comparatively much less rapid.

2. *Release of fatty acids.* In contrast to the ease of obtaining fat deposition *in vivo*, the demonstration of fat release from adipose tissue *in vitro* met with considerable difficulties. When adipose tissue was incubated *in vitro* in various protein-buffer solutions, only negligible amounts of fatty acids and glycerides were released into the medium. This difficulty can be understood if it is remembered that the removal of molecules from the lipid medium of the adipose cell into the aqueous external medium proceeds against the direction of thermodynamic equilibrium. It was considered therefore as likely, that fatty acids and triglycerides, to be able to leave the cell, have to be bound to a serum protein in order to shift the equilibrium. When several media were tried, it was found that only media containing serum albumin supported fatty acid release (Reshef, Shafrir, and Shapiro, 1956) (see Table 4).

Table 4. Release of unesterified fatty acids from adipose tissue

According to Reshef, Shafrir, and Shapiro (1956)

Mesenteric adipose tissue of rats fasted for two days were incubated at 37°C in 3 ml of a Krebs-Ringer phosphate solution containing 5% bovine serum albumin. Fatty acids were extracted from the medium with hot alcohol ether and titrated in alcoholic solution after thorough washing with water.

	Fatty acids in microequivalents found in medium		
	After 5 min	*After 3 hr*	*Δ5 min–3 hr*
Full medium . . .	0·65	2·3	1·65
	1·1	2·9	1·8
	1·65	3·2	1·55
Albumin omitted . .	0·65	0·7	0·05
	0·85	0·65	− 0·20
	1·10	1·10	0
	0·24	0·60	0·36

The mobilization of fatty acid esters (triglycerides?) in all these cases was very slow. The carrier protein for this release could be ascertained only by labelling the tissue fatty acids and esters by preincubation *in vitro* with radiostearic acid, according to Shapiro *et al.* (1955). When the labelled tissue was then transferred to a fresh medium, containing serum or serum albumin, labelled fatty acids could be detected on a paper electrophoretogram, moving with the albumin fraction, while small amounts of radioactivity are found in the β-lipoprotein. Part of these latter fatty acids can be shown by chromatography on a MgO column to be esterified fatty acids.

Since the amount of unesterified fatty acids (UFA) released into the albumin medium exceeded the amount of UFA preformed in the tissue, the

liberation of fatty acids from the tissue involves the splitting of fatty acid from the triglycerides in the tissue. This may be performed by a tissue lipase, the presence of which in adipose tissue has been shown repeatedly (QUAGLIARIELLO and SCOZ, 1932; RENOLD and MARBLE, 1950) or by a lipoprotein lipase recently demonstrated by KORN and QUIGLEY (1955). Another possibility may be visualized in the form of reversal of the enzymatic reactions leading to the uptake of fatty acids by the tissue and their esterification. Although no such reversible reaction has so far been demonstrated, this alternative has the attraction of providing a single mechanism for the mobilization and deposition of fatty acids.

The results obtained by the *in vitro* studies, that unesterified fatty acids are an important vehicle for the transport of fat into and out of adipose tissue, acquired further significance through the findings of GORDON and CHERKES (1956) that the amount of unesterified fatty acids increased in blood plasma under conditions of fat deposition as well as of fat mobilization. The values of UFA tended to rise after ingestion of fat and also on fasting. Epinephrine injection (which has been shown to accelerate fat mobilization) likewise caused an increase in UFA in blood.

The finding of an efficient mechanism for the assimilation of fatty acids is complementary to previous knowledge of the behaviour of fat in the circulation. After absorption from the intestine, fat appears in the circulation as small droplets, named chylomicrons. After some time these chylomicrons disappear and the plasma becomes clear again. Blood serum was found to contain a "clearing factor" which is activated by heparin adminstration (HAHN, 1943). The principal product of the clearing reaction was found to be unesterified fatty acids (GORDON, BOYLE, BROWN, CHERKES, and ANFINSEN, 1953). It is highly likely that most of these acids are removed from blood by adipose tissue with the aid of the mechanism postulated above.

The formation of free fatty acids seems to be an important preparatory step for deposition as well as for mobilization of fat. However, the results discussed point to a second alternative mechanism by which unsplit triglycerides are assimilated.

It remains to be shown how the various factors which accelerate the transport act on the adipose cell and on its enzymes. Some results are available which show that the lipase activity of adipose tissue increases when fat mobilization is accelerated, as in hunger and diabetes (HAUSBERGER and BUBLITZ, 1949). On the other hand, nothing is known on the regulation of the activity of the other cell factors participating in lipid transport.

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8

DILATOMETRY

B. M. Craig

CONTENTS

		Page
I.	INTRODUCTION	198
II.	VOLUME-TEMPERATURE RELATIONS	199
III.	CONSTRUCTION AND OPERATION OF DILATOMETERS	200
	A. Errors in dilatometry	200
	B. Type of dilatometer	201
	C. Type of bulb	202
	D. Confining liquid	203
	E. "Apparent contractions"	203
	F. Pretreatment of samples	204
IV.	FATTY ACIDS	204
V.	METHYL ESTERS OF FATTY ACIDS	205
VI.	MONO- AND DI-GLYCERIDES	209
VII.	TRIGLYCERIDES	210
VIII.	SYNTHETIC GLYCERIDE MIXTURES	212
IX.	POLYMORPHISM	215
	A. Tristearin	215
	B. Monostearin	217
	C. Stearic acid	217
	D. Acetostearins	218
	E. Tung oil	218
	F. Cocoa butter	219
X.	NATURAL PRODUCTS	219
XI.	DETERMINATION OF SOLIDS CONTENT	223
XII.	CONCLUSIONS	225
	REFERENCES	225

8

DILATOMETRY

B. M. Craig

I. Introduction

Dilatometry or the study of volume-temperature relationship has been used in inorganic chemistry to study the change in volume with temperature for solutions of inorganic salts; in organic chemistry to measure volume expansion of organic liquids; in rubber chemistry to study changes in structure of rubber under the influence of temperature, and to study phase changes of materials in the solid state. The dilatometer has been employed in the field of fats and oils to measure expansivities of solid and liquid states and the volume change on melting of various fatty acids, esters, and natural products. Some studies have been directed to investigations of phase changes of glycerides and fatty acids by measuring volume change with temperature for the various polymorphic forms.

Dilatometry is directly related to calorimetry[10] in that the former requires a measurement of volume upon application or removal of heat whereas the latter requires a measure of heat content at various temperatures. Heat content plotted against temperature will give the relation in solid and liquid states which compares to expansivities in the same states as measured with the dilatometer. The heat of fusion then corresponds to the melting dilation. However, dilatometry which is a static procedure as compared to the dynamic procedure used in calorimetry has two advantages:

(1) simpler equipment is required;
(2) adequate time can be allowed to attain equilibrium.

This is a definite advantage in studying polymorphic transformations or like studies where much time may be required.

Unfortunately too little research in the fats and oils field has been devoted to studies of pure compounds and mixtures of pure compounds. This is particularly true in the study of triglycerides where the major amount of work has dealt with natural fats and oils, partially and completely hydrogenated fats and oils, and commercial products such as shortenings and margarine oils. This has resulted in erroneous deductions and interpretations of physical behaviour which are gradually being eliminated by more fundamental work on known compounds and mixtures. The dilatometer is finding increased favour with the manufacturers of shortenings and margarine oils, and appears to give reliable results where the measurements are made on a relative basis. More fundamental work on materials such as the isoacids and glycerides produced

during hydrogenation will undoubtedly lead to more quantitative measurements. The principal industrial use of the dilatometer is to obtain a measure of the solids content of shortenings and margarine oils. This measurement is more properly being referred to now as the "solids content index" since the values are approximations to the amount of solid glycerides which may be present in the material under study.

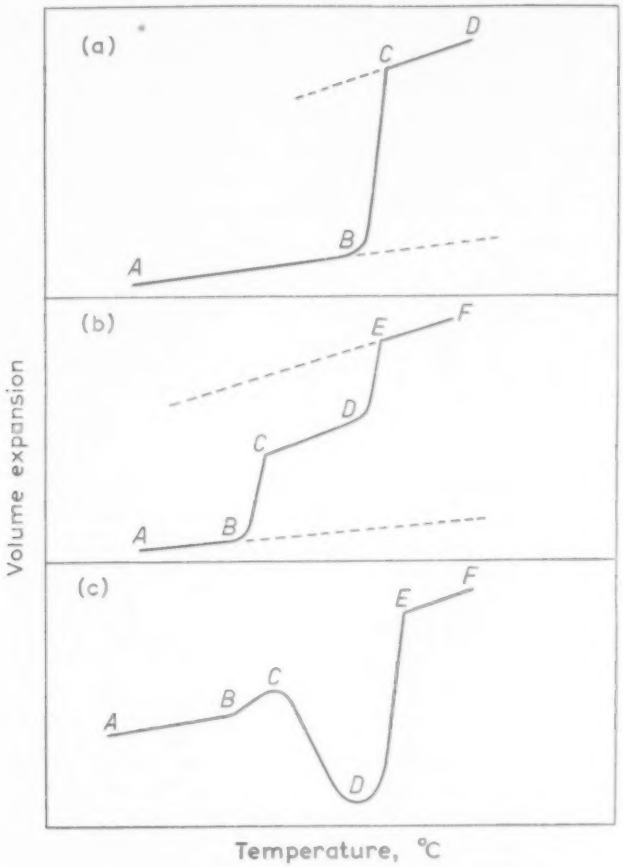


Fig. 1. Dilatometric curves expected for a single compound [a], a mixture of two compounds [b], and a compound in an unstable polymorphic form [c].

II. Volume-Temperature Relations

The general types of volume-temperature relations which would be anticipated for simple systems are illustrated in Fig. 1. The dilatometric curve for a single pure compound is shown in Fig. 1(a). The linear section AB represents the change in volume with temperature for the solid state. The slope of this linear section is the coefficient of expansion for the solid state. Section BC represents

melting of the compound and section CD the expansivity in the liquid state. The perpendicular distance from the melting point at C to the extension of the solidus line is the volume change on melting or melting dilation. The dilatometric curve shown in Fig. 1(c) illustrates a single pure compound which was obtained in an unstable polymorphic form. The expansion of the solidus state is followed by partial melting at which point the more stable polymorphic modification is obtained. The dilatometric curve then follows the type which would be expected for the more stable form. Fig. 1(b) illustrates the dilatometric curve for a mixture of two pure compounds which have melting points sufficiently far apart to be detected by dilatometric measurements. The dilatometric curve appears as two single compounds each having the form shown in Fig. 1(a). The section CD will be made up of the combined expansivities of liquid and solid, and section EF will be the combined expansivities of both components in the liquid state. The melting dilations for each compound will be related to the amount of that compound which was present in the mixture.

III. Construction and Operation of Dilatometers

Dilatometers may be constructed either from vitreous silica or Pyrex glass; the former has a much lower coefficient of expansion than the latter. A number of investigators have found that dilatometers made of Pyrex glass require some use before becoming sufficiently well tempered to yield reproducible expansions and contractions. This is important only where precise measurements are being made. The coefficients of expansion for glass have been very well investigated.[1–5]

A. Errors in dilatometry

The following points are important in dilatometric measurements:

(1) Complete removal of air from the ester, glyceride, or fatty acid which is to be placed in the dilatometer. Fats and oils are capable of absorbing relatively large quantities of air, some of which will be released as the material is transformed from the liquid to the solid state. The presence of air leads to a larger figure than is correct for coefficients of expansion in the solid and liquid states. The air is readily removed by the application of a vacuum and violent agitation while the fat is maintained alternately in liquid state and solid states.

(2) Removal of air from the confining liquid. This is particularly important where water is used.

(3) Volume correction for expansion or contraction of the confining liquid.

(4) Volume correction for the expansion or contraction of the glass of the dilatometer.

(5) "Stem emergent" correction for volumetric dilatometers. This correction may also have to be applied to gravimetric dilatometers unless very small bore capillary is used. The relation of the weight of mercury in the exposed stem to the weight of mercury in the dilatometer bulb may be used to determine the need for such a correction.

(6) Control of the bath temperature. The degree of control of bath temperature depends on the precision required in the measurements. An approximate calculation of the effect of variation in bath temperature may be made in the following manner. If the investigator is interested in the expansivities of the solid and liquid states one may assume figures of 0·000,25 and 0·000,95 ml/gm/°C respectively for coefficients, and approximately 100 gm of mercury in the dilatometer. A temperature variation of ± 0·01°C will give a maximum error

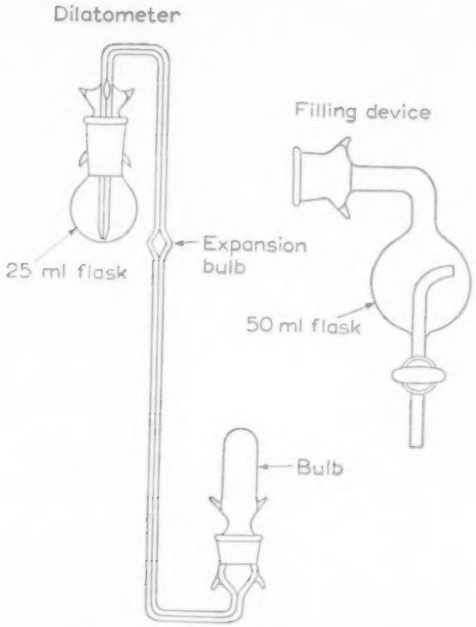


Fig. 2. Gravimetric dilatometer and filling device.[19]

in mercury volume of 100 × 0·01 × 0·000,0134 or ± 0·000,01 ml. If the sample in the dilatometer bulb weighs 5 gm the error would be ± 0·000,002. Similarly the error in the sample would be ± 0·01 × 0·000,25 or ± 0·000,002 ml. The maximum error in coefficient for the solid state as affected by temperature variation in the control bath would be (0·000,004)2 = 0·000,008 ml/gm/°C. By a similar procedure the maximum error to be expected for the coefficient in the liquid state would be 0·000,02 ml/gm/°C.

B. Type of dilatometer

Dilatometers used by early workers were of the volumetric type. NORMANN[6] constructed a volumetric dilatometer which consisted of a cylindrical glass bulb to the bottom of which was sealed a length of capillary tubing. The top of the bulb was provided with an opening for filling the bulb. The top of this opening was ground flat and was sealed by a piece of optically flat glass held in place by

a yoke and screw clamp. HOFGAARD[7] used a similar type of dilatometer with a modification in the device for sealing the opening. BAILEY and KRAEMER[8] used a volumetric dilatometer in which the dilatometer bulb was connected to the capillary tube by a ground-glass joint, and a stopcock was provided at the top of the bulb to facilitate filling of the bulb. A volumetric type which has been used in industry is described by FULTON, LUTTON, and WILLE,[9] and a number of laboratory supply companies are marketing volumetric dilatometers.

The gravimetric type of dilatometer is recommended for more precise measurements. The model designed by BAILEY and KRAEMER[8] is a modification of

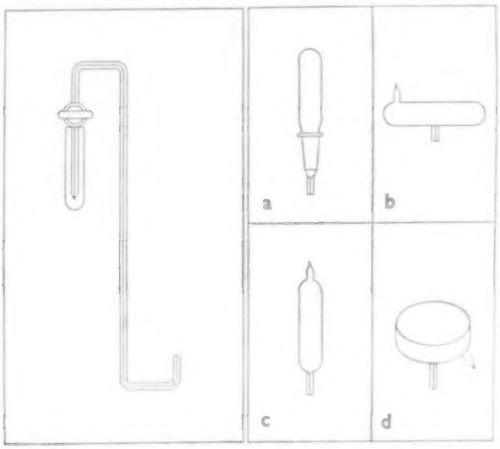


Fig. 3. Different types of dilatometer bulbs.

that used by BURLEW,[3] and is an all-glass sealed unit wherein all possibilities of leakage are eliminated. The model illustrated in Fig. 2 is a modification[19] of that used by BAILEY and KRAEMER and makes use of a ground-glass joint between the bulb and capillary tubing. However there is a possible error in the use of the ground-glass joint in both leakage and a difference in volume of the dilatometer due to improper seating. This model has been further modified as shown in Fig. 3(c) to the use of a 6-mm tube sealed to the top of the dilatometer bulb to facilitate filling of the bulb and evacuation of the sample. The capillary tube was 0·5-mm bore to make the "stem-emergence" corrections negligible. The use of such fine-bore capillary tube did not permit efficient evacuation of the sample with the filling device used by BAILEY and KRAEMER[8] (Fig. 2).

C. Type of bulb

Most research workers in the fats and oils field appear to have used dilatometer bulbs which are made in the form of cylinders. Some work has been done in the author's laboratory on effect of the size and shape of bulb on the rate of melting of samples. A cylindrical unit of 14·5-ml capacity and 1·5-cm diameter

was compared to a cylindrical unit of 5·0-cm diameter as illustrated in Fig. 3(c) and (d). Five grams of methyl palmitate was placed in each bulb. After equilibration, dilatometric measurements were made, and at 30·0°C the sample in the 5-cm diameter bulb melted in 4 hr whereas the sample in the 1·5-cm diameter bulb required 24 hr to melt completely. The difference in time required for complete melting has not been explained, but is believed to be due to a difference in heat transfer coefficient across one of the interfaces, e.g. glass-mercury, mercury-fat, etc. Furthermore it has been found that the glass correction also depends on the shape of the bulb—the flat-shaped bulbs will have a correction figure around 90 per cent per unit volume of the corresponding cylindrical bulb.

D. Confining liquid

A confining liquid is used in dilatometric studies and should have the following characteristics:

(1) Be completely immiscible with the material under consideration.

(2) Have a known density or specific volume at all the temperatures to be used in the study.

Water, alcohol-water, and mercury are the liquids which are most generally used. The alcohol-water mixture was used by BERTRAM[10] because of its low surface tension. Water has been used most generally in the volumetric type of dilatometer. Mercury has been used in both volumetric and gravimetric dilatometers, but has the disadvantage of a high interfacial tension with fats which prevents mercury from penetrating small crevices in the fat sample. However, mercury has two advantages; (*a*) complete immiscibility with fat or fatty compounds, and (*b*) the density of mercury allows a more accurate determination of volume change when used in gravimetric dilatometers. The use of water as a confining liquid limits measurements to temperatures between 0°C and 100°C whereas mercury may be used to its melting point — 38·87°C. It would be advantageous to be able to make dilatometric measurements to temperatures below — 38·87°C, but to date no satisfactory confining liquid has been found. The densities of both water and mercury have been very accurately determined and may be found in International Critical Tables[11] or similar compilations.

E. "Apparent contractions"

Volume decreases have been noted in the temperature region preceding the melting point by a number of workers.[7, 12, 19, 20] These have occurred principally in investigations which have involved fully saturated fats. Various terms such as vacuole formation and after-contractions have been applied to these phenomena. BAILEY and his co-workers have suggested that the volume contractions are due to minute cracks or crevices in the fat into which the mercury cannot penetrate. Such cracks may be readily discerned in a sample of solid fat either visually or with the aid of a low-power microscope. It is possible that when the temperature of the sample approaches the melting point

expansion acts to close the cracks. This expansion is not reflected in an expulsion of mercury from the dilatometer bulb and the resultant effect is an "apparent contraction." While this explanation appears very feasible and easily explains the phenomenon it has not been definitely proved.

The difficulty was resolved by BAILEY and his co-workers[12] by forming the fat into small pellets to allow a more intimate contact between the mercury and the fat. CRAIG[19] formed the samples into solid rods, and VAECK[20] used a long equilibration time to overcome the difficulty.

The errors in dilatometric measurements may all be readily estimated, those due to temperature control, glass volume correction, stem emergent correction, confining liquid correction, weight and size of sample, and weight of confining liquid. For very precise work the magnitude of individual errors may then be reduced by control of size of sample, type and size of dilatometer bulb, and size of capillary for volumetric dilatometers. In this way more reliability can then be placed on the results, and these results can be compared directly to those obtained in other laboratories.

F. Pretreatment of the sample

This factor is important in pure materials since the pretreatment influences the polymorphic form which may be obtained. Differences have been found in the dilatometric curves for natural and derived products which had been chilled slowly or quickly (Fig. 16). The explanation of this phenomenon may lie in polymorphic form or crystal size or both and positive proof for the effect has not been obtained.

IV. FATTY ACIDS

GARNER and RYDER[13] measured the melting dilations of a number of the lower members of the fatty-acid series including both even- and odd-numbered acids. These workers used volumetric dilatometers in which air and mercury were the confining materials. The authors realized the effect of solution of air and did not report expansivities for the solid and liquid states. The data are included in Table 1 with the more comprehensive data obtained by SINGLETON and his co-workers.[14–17]

SINGLETON and co-workers used gravimetric dilatometers to study the saturated-acid series from caproic to stearic, and obtained the following equation relating melting dilation in millilitres per mole to the chain length; $V = (n - 2)\ a + b$ where n is the number of carbon atoms in the chain and a and b are constants, $a = 3{\cdot}57$ and $b = -3{\cdot}24$. The equation is valid only for the saturated fatty acids with more than ten carbon atoms in the chain, and is illustrated in Fig. 7. The influence of the carboxyl group becomes relatively greater in fatty-acid molecules with less that ten carbon atoms, and the presence of an unsaturated linkage results in lower values for melting dilations. SINGLETON and WARD[17] studied the effect of unsaturated linkages on melting dilations and expansivities in oleic, elaidic, petroselinic, and petroselaidic acids. The data

are included in Table 1 and the melting dilations are all seen to be lower than for stearic acid. The trans isomers show higher melting dilations than the corresponding cis isomers. The melting dilation of petroselinic acid is higher than that of oleic acid, and indicates that the proximity of the unsaturated linkage to the carboxyl group increases the melting dilation. Further research on the effect of the position of the unsaturated linkage in the fatty-acid chain is required to establish a general rule. However, general relations may be deduced from the heats of melting since these have been shown to be directly related to dilatometric results.[10]

Table 1. Melting dilations and expansivities of fatty acids

Fatty acid	*Reference*	*Melting dilation*		*Expansivity ml/gm/°C*	
		ml/gm	*ml/mol*	*Solid*	*Liquid*
Even-numbered					
Caproic	(15)	0·116	13·5	0·00035	0·00098
Caprylic	(13)	0·119	17·2	—	—
Capric	(13)	0·130	22·4	—	—
Capric	(15)	0·124	21·4	0·000329	0·000984
Lauric	(13)	0·143	28·6	—	—
Lauric	(15)	0·166	33·2	0·000190	0·000963
Myristic	(15)	0·172	39·3	0·000191	0·000972
Palmitic	(15)	0·181	46·4	0·00028	0·00097
Stearic B	(16)	0·2047	58·6	0·00027	—
C	(16)	0·186	52·9	0·00026	0·00097
Oleic	(17)	0·1183	33·4	0·000269[a] 0·000308[b]	0·000957
Elaidic	(17)	0·1615	45·6	0·000253	0·000968
Petroselinic	(17)	0·1314	37·1	0·000369	0·000895
Petroselaidic	(17)	0·1403	39·6	0·000308	0·000900
Odd-numbered					
Nonylic	(13)	0·086	13·6	—	—
Undecylic	(13)	0·108	20·1	—	—

[a] Measured below − 5°C.
[b] Measured − 4°C to 10°C.

V. Methyl Esters of Fatty Acids

Some work on volume-temperature relations for methyl esters has been done by the author,[18] and the results are being presented here. Gravimetric dilatometers similar to those used by Bailey and his co-workers[8] were used to study methyl palmitate, methyl stearate, methyl arachidate, methyl behenate, and methyl oleate.

The expansivities and melting dilations are presented in Table 2. The melting dilations of the saturated esters in millilitres per mole plotted against number of carbon atoms in the molecule give a reasonably straight line relationship. The melting dilation of methyl oleate is unexpectedly high when compared to melting dilations reported for triolein,[12, 19] whereas the melting dilations of

methyl palmitate and methyl stearate are comparable to tripalmitin and tristearin.

A change in the slope of the solidus line for the triglycerides has been referred to by BAILEY and SINGLETON[12] and by CRAIG and co-workers.[19] This was further investigated by CRAIG[18] in a study on methyl palmitate and methyl stearate. The expansivities of three samples of methyl palmitate and four samples of methyl stearate were measured from — 38° to 1·0 and 1·5° respectively

Table 2. Melting dilations and expansivities of methyl esters of fatty acids

	M.pt. °C	*Melting dilation*		*Expansivity ml/gm/°C*	
		ml/gm	*ml/mol*	*Solid*	*Liquid*
Methyl palmitate	30·1	0·1698	45·92	0·00025	0·00099
Methyl stearate	39·9	0·1726	51·52	0·00022	0·00100
Methyl arachidate	46·9	0·1769	57·76	0·00025	0·00101
Methyl behenate	53·3	0·1821	64·57	0·00022	0·00100
Methyl oleate	— 19·8	0·1187	35·19	0·00036*	0·00090

* 0·00036 ml/gm/°C was found for the temperature interval from — 38° to — 26°C; the coefficient 0·00030 was used to calculate the melting dilation.

Table 3. Melting dilations and expansivities of mono- and di-glycerides

Glyceride	*Melting dilation*		*Expansivity ml/gm/°C*	
	ml/gm	*ml/mol*	*Solid*	*Liquid*
Monocaprylin (15)	0·052	11·3	0·000204	0·000736
Monolaurin (15)	0·110	30·2	0·000341	0·000956
Monomyristin (15)	0·118	35·7	0·000345	0·000844
Dilaurin (15)	0·126	57·5	0·000391	0·000874
Dimyristin (15)	0·141	72·3	0·000435	0·00100
Mono-olein (15)	0·067	24·0	0·00034	0·00081
Monostearin:				
Beta form (23)	0·1367	49·0	0·000241	0·000920
Beta prime form (23)	0·0610	21·9	0·000291	0·000920
Sub-alpha form (23)	0·1292	46·3	0·000303	0·000920

below the melting point in four successive runs without melting the samples. The cooling cycles were likewise measured between each heating cycle and the averages of these determinations are presented in Fig. 4 and 5. An accurate determination of the "breakpoint" in the solidus line was obtained in this manner. This occurred at — 19°C for methyl palmitate and — 10°C for methyl stearate or 49° below the melting point of the respective methyl esters. CRAIG, LUNDBERG, and GEDDES[19] observed breakpoints around 50° below the melting points of the saturated glycerides. Since these were single determinations, the actual breakpoints may lie at some lower temperature. The present work on

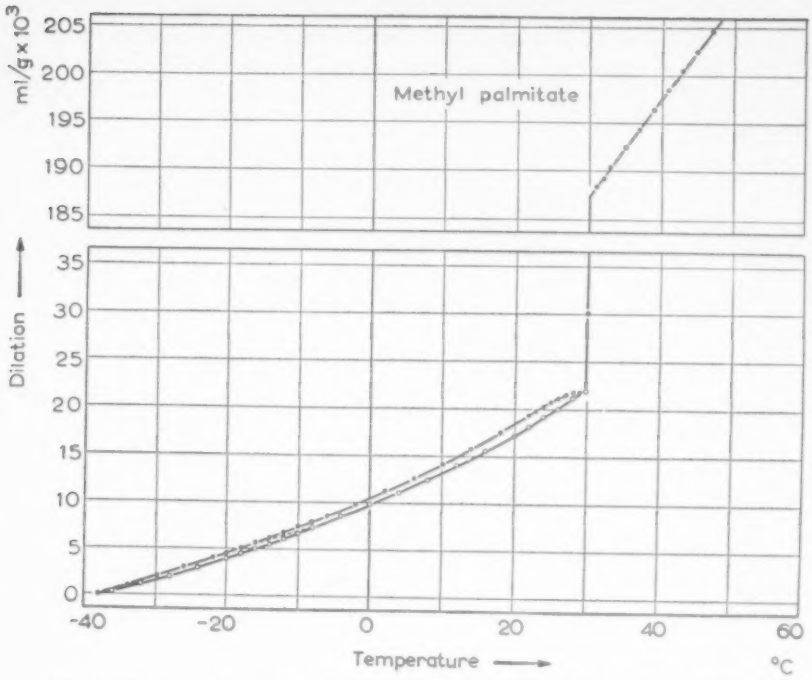


Fig. 4. Dilatometric curves for heating and cooling curves of methyl palmitate.[18]

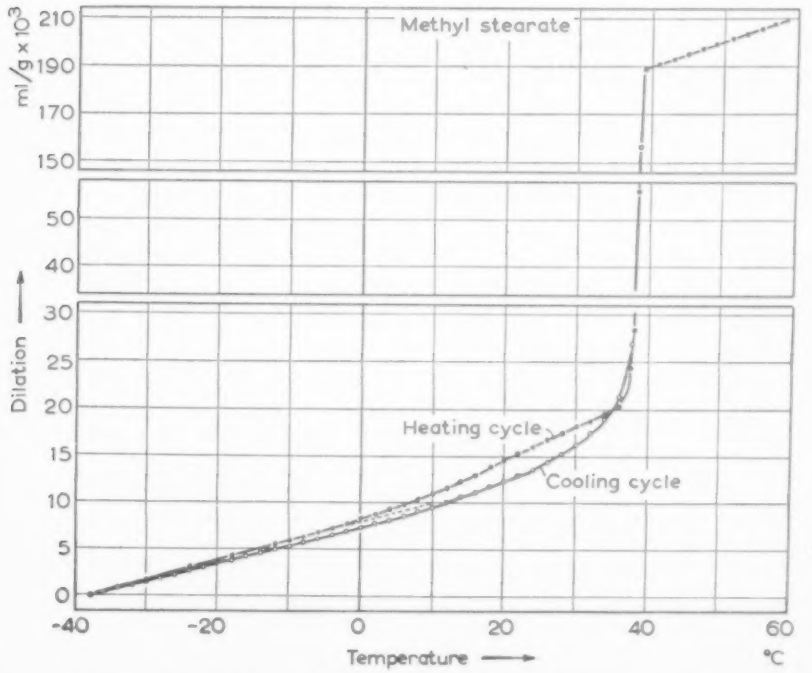


Fig. 5. Dilatometric curves heating and cooling cycles of methyl stearate.[18]

methyl esters shows a curvilinear relationship from the breakpoint temperature to the melting point. CRAIG, LUNDBERG, and GEDDES[19] gave a linear coefficient for the volume-temperature relation in this region, but had insufficient data for an accurate determination of either linearity or the breakpoint An explanation for this phenomenon is not immediately apparent, but the phenomenon is important in determining the coefficient of expansion in the solid state which influences the magnitude of the melting dilation. This is particularly important in measurements involving materials which have melting points lower than 15°C, i.e. unsaturated fatty-acid esters and glycerides.

It has been shown that certain linear long-chain molecules exhibit an X-ray pattern[21] and dielectric measurements[22] in the melting region which have been interpreted as due to the ability of the molecules to exert rotational vibrations in the lattice structure. The explanation is in accord with the modern theory of melting. The change of slope of the solidus line found in dilatometric studies may be related to this phenomenon. Two nonlinear compounds, diphenyl methane M.pt. 25·5°C and diphenyl amine M.pt. 52·5°C were studied with the dilatometer. A breakpoint was found for diphenyl amine at around 0°C and the data indicated a breakpoint for diphenyl methane at around — 25°C, so the phenomenon is apparently not confined to long-chain linear compounds. The volume-temperature relation for diphenyl methane was curvilinear at temperatures between the breakpoint and the melting point, but heating and cooling curves appeared to be identical.

An interesting phenomenon has been noted in the temperature region within a degree of the melting point. This is illustrated by reference to Fig. 5, where equilibrated volumes are shown to within 0·1° of the melting temperature of methyl stearate. The dilatometer has been suggested[10] for very accurate determinations of melting point by measuring two points on the vertical melting section and two or more points on the liquidus line. The intersection of these two lines gives the final melting temperature to the second decimal place. The present data show that the determination of the two points on the vertical section must be made with extreme care. Since at least part of the vertical section is curvilinear, the position of these points must be close to the final melting temperature and the temperature of the bath must be carefully controlled, since V/T is very large in this temperature region. It should also be noted that an equilibrated point was obtained for the methyl stearate at 39·0°C which represented most of the solid converted to the liquid state. Further equilibration for three days produced no measurable change in volume. This point represents a stable state which may or may not have been a true state of equilibrium. If the present data were accepted as correct it is fairly obvious that methyl stearate must melt over a range of temperature, the lower limit of which may arbitrarily be taken at any temperature between the breakpoint and the final melting temperature.

The problem of volume decreases or contraction has been noted by HOFGAARD,[7] BAILEY *et al.*,[12] CRAIG *et al.*,[19] and VAECK,[20] and has been discussed in a previous section. It is apparent from Figs. 4 and 5 that the heating and

cooling curves were not the same for methyl palmitate and methyl stearate. The cooling cycle was completely curvilinear from 29 to — 38°C for methyl palmitate and from 37·5 to — 38°C for methyl stearate. Whether this phenomenon is a hysteresis effect has not been investigated. The most interesting point is that the total volume change from — 38 to 29°C and from — 38 to 37·5°C was the same for both the heating and cooling cycles. These data would appear to indicate that the "after-contraction" was a real and reproducible phenomenon, but further research is required to prove the point. It is difficult to explain the

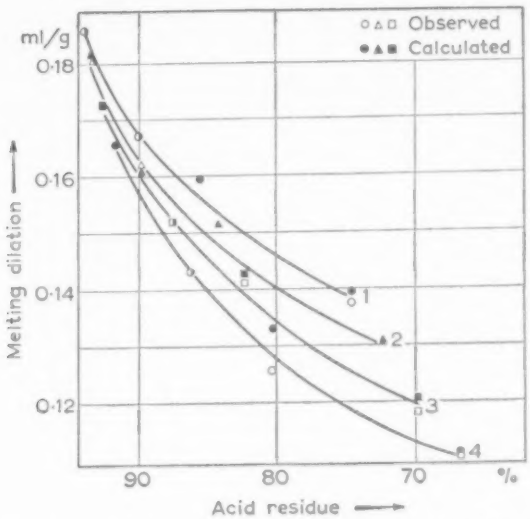


Fig. 6. Relation between melting dilations and per cent of acid residue glycerides.[15] (1) stearyl, (2) palmityl, (3) myristyl, and (4) lauryl.

nonlinearity of the cooling cycle between the breakpoint and — 38°C for the particular ester.

VI. Mono- and Di-glycerides

Singleton and co-workers[15, 23] measured the melting dilations and expansivities of a number of mono- and di-glycerides which are given in Table 3. Equations were developed[15] relating melting dilations on a mole basis with the number of carbon atoms in the fatty-acid radical as follows:

Monoglycerides	$V = (n - 2)a + b$	$a = 3{\cdot}24$	$b = -2{\cdot}08$
Diglycerides	$V = 2((n - 2)a + b)$	$a = 3{\cdot}24$	$b = -2{\cdot}08$

The equations are valid only for the saturated acids with more than ten carbon atoms in the chain. Singleton *et al.* found that by multiplying the melting dilation of a particular fatty acid by the relative content of that acid in the glyceride, i.e. 74·6 per cent for monoglycerides, 90 per cent for triglycerides, they obtained calculated values for the melting dilations of the particular

glycerides which were in accord with the observed values. The end groups (e.g. methyl and carboxy) were thus exerting the same influence on melting dilations, and the differences in an homologous series were the result of the difference in the number of methylene groups, i.e. two CH_2. Monoglycerides had one half the melting dilation of diglycerides and one third that of triglycerides on a molar basis. The same relation holds for derivatives of oleic acid, where the melting dilation of mono-olein is 24·0 ml/mol as compared to 73·5 ml/mol for triolein.

The relation between melting dilations as millilitres per gram and per cent acid residue for lauric, myristic, palmitic, and stearic acids and their glyceryl esters shown in Fig. 6, and the relation between the melting dilation on a molar

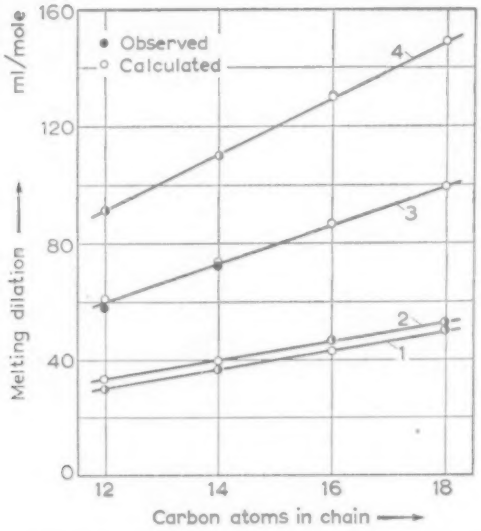


Fig. 7. Relation between melting dilations in millilitres per mole and number of carbon atoms in the fatty acid for fatty acids, mono-, di-, and tri-glycerides.[15] (1) monoglycerides, (2) acids, (3) diglycerides, (4) triglycerides.

basis and the number of carbon atoms in the chain for the mono- and di-glycerides, is shown in Fig. 7.

VII. Triglycerides

More research on volume-temperature relations has been applied to triglycerides than the fatty acids or esters of monohydric alcohols due to the occurrence of these compounds in natural products. Bailey and Singleton[12] studied the melting dilations and expansivities of a number of triglycerides. Singleton and Gros[15] developed an equation relating molar dilations to chain length of the fatty acids $V = 3(n - 2)a + b$ where $a = 3{\cdot}24$ and $b = -2{\cdot}08$. The data are given in Table 4 and the relation of molar melting dilation and chain length is represented in Fig. 7. The coefficients of expansion for the solid state were

calculated at temperatures from — 38 to around — 25°C for trilaurin, trimyristin, tripalmitin, and tristearin where BAILEY and SINGLETON judged the glycerides to be completely in the solid state. The coefficient for triolein was assumed to be around 0·00030 ml/gm/°C as the observed coefficient was believed to be too high. This is justified on the same basis as the relations described in the section on methyl esters. CRAIG, LUNDBERG, and GEDDES[19] studied the volume-temperature relations for a number of simple and mixed symmetrical triglycerides of oleic, palmitic, and stearic acids. The data are included in Table 4 with values for melting dilations and expansivities in the solid and liquid states. The data presented here have been recalculated by the author to be more in line with the data presented by BAILEY and co-workers. The dilatometric measurements by CRAIG *et al.* were started in most cases at — 15°C

Table 4. Melting dilations and expansivities of triglycerides

Triglyceride	*M.pt. °C*	*Melting dilation*		*Expansivity ml/gm/°C*	
		ml/gm	*ml/mol*	*Solid*	*Liquid*
Trilaurin (12)	46·5	0·1428	91·24	0·00019	0·00090
Trimyristin (12)	56·6	0·1523	110·13	0·00021	0·00091
Tripalmitin (12)	65·5	0·1619	130·70	0·00022	0·00092
(19)	65·2	0·1582	127·71	0·00027	0·00097
Tristearin I (12)	72·5	0·1671	148·97	0·00023	0·00092
(19)	72·0	0·1610	143·53	0·00026	0·00095
Tristearin II (12)	64·5	0·1316	117·32	0·00029	—
Tristearin III (12)	54·5	0·1192	106·26	0·00032	—
Trielaidin (12)	42·0	0·1180	104·48	0·00018	0·00087
Triolein (12)	4·6	0·083	73·49	0·00030†	0·00082
(19)	4·8	0·0782	68·17	0·00030†	0·00093
(19)	4·8	0·0796	69·06	0·00030†	0·00099
Stearodiolein (19)	23·5	0·1178	101·78	0·00030†	0·00105
Oleodistearin (19)	42·5*	0·1106	94·28	0·00030†	0·00092
Oleodipalmitin (19)	35·5	0·1240	100·32	0·00030†	0·00091
Palmitodistearin (19)	67·7	0·1553	134·09	0·00026	0·00093
Stearodipalmitin (19)	67·7	0·1527	127·55	0·00027	0·00097

* Existed in unstable polymorphic form.
† 0·00030 used to calculate melting dilation.

and in the light of the subsequent work on the methyl esters there is room for a considerable error in determining the coefficients of expansion in the solid state. Thus the recalculation has involved using coefficients for the solid state obtained on the saturated glycerides to 0°C, and assuming a coefficient of 0·00030 ml/gm/°C for unsaturated glycerides as was used by BAILEY *et al.* for triolein. The coefficients of expansion of both solid and liquid states are slightly higher than obtained by BAILEY *et al.*[12] which might indicate an incomplete removal of air from the samples. The melting dilation for oleodistearin is somewhat low, and this could be attributed to a polymorphic form. It was found difficult to obtain the most stable form of this glyceride even in a capillary tube, and the data shows that there was melting at 38·5°C (M.pt. of beta prime form).

The data for stearodiolein, oleodistearin, and palmitodistearin are given in Fig. 8 to show the general type of volume-temperature relationships which were obtained.

The main point of interest is the break in the line representing expansion in the solid state for the saturated triglycerides which may also be observed in

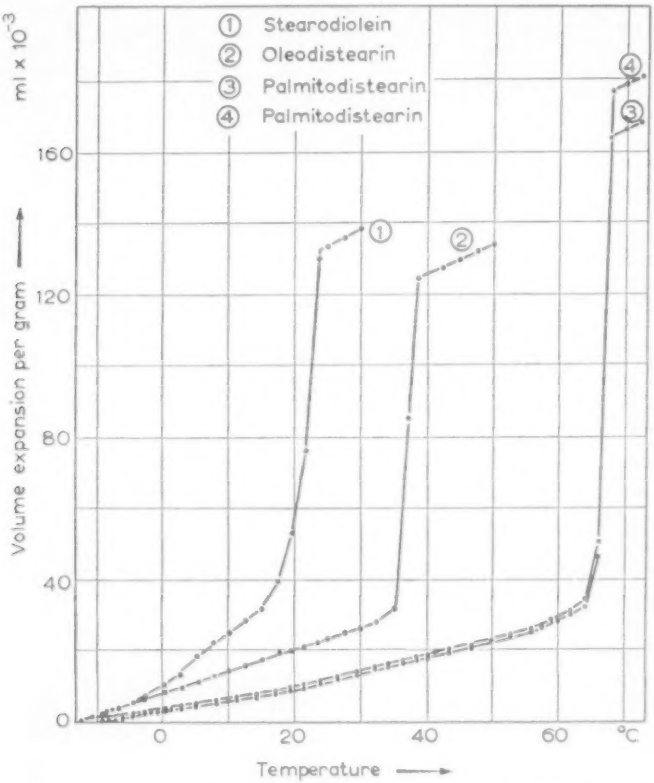


Fig. 8. Dilatometric curves for stearodiolein, oleodistearin, and palmitodistearin.[19]

data obtained by Bailey *et al.*[12] The breakpoint occurred around 45–50° below the melting point of the particular glyceride. Trielaidin has a higher melting dilation than triolein, but both are considerably lower than tristearin, indicating the effect of unsaturation, and the effect of cis-trans isomerism.

VIII. Synthetic Glyceride Mixtures

The volume-temperature relations of binary, ternary, quaternary, and octernary mixtures of the synthetic glycerides triolein, tristearin, tripalmitin, stearodiolein, oleodistearin, oleodipalmitin, stearodipalmitin, and palmitodistearin were studied by Craig, Lundberg, and Geddes.[19] The dilatometric curve for a binary mixture composed of one-third tristearin and two-thirds triolein showed

that the triolein had the same melting point as the single glyceride and each component showed discrete melting segments. Similar discrete melting segments were observed in other simple mixtures such as triolein-tripalmitin, triolein-stearodiolein-palmitodistearin. The volume-temperature relations for

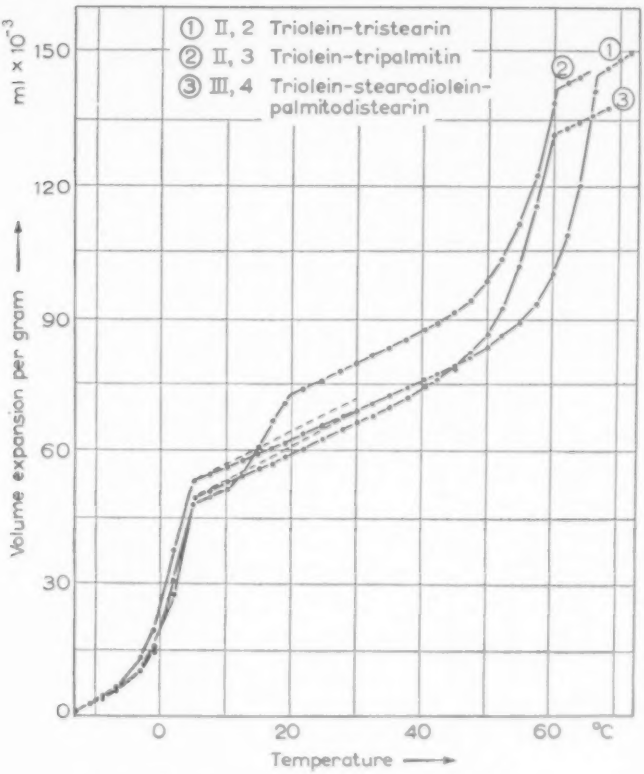


Fig. 9. Dilatometric curves for triglyceride mixtures.[19]
II. (2) triolein 66·5%, tristearin 33·5%.
II. (3) triolein 66·5%, tripalmitin 33·5%.
III. (4) triolein 57·4%, stearodiolein 25·2%, palmitodistearin 24·4%.

some of these compounds is shown in Fig. 9. When the complexity of the mixture was increased or components of the mixture had melting points which were closer together than 15°C, the discrete melting segments disappeared and were replaced by smooth curves. An example of this is shown in Fig. 10, where curve 2 composed of triolein-stearodiolein-oleodipalmitin-tristearin shows that the melting segment of stearodiolein has merged with that of oleodipalmitin. Curve 3 is composed of a similar quaternary mixture, but oleodistearin (M.pt. 42·5°) is substituted for oleodipalmitin (M.pt. 35·5°). The resultant dilatometric curve may have inflection points between 5° and 40°, but these are difficult to detect. Inflection points may be observed in curves 2, 3, and 4 preceding the

melting of the fully saturated glycerides. Curve 1 is a quaternary mixture in which only the triolein appears as a discrete melting segment, the melting of the other three glycerides being blended into a smooth curve with no inflection points. It would appear from this and like data presented by CRAIG *et al.*[19] that discrete melting segments are shown only where melting points of the

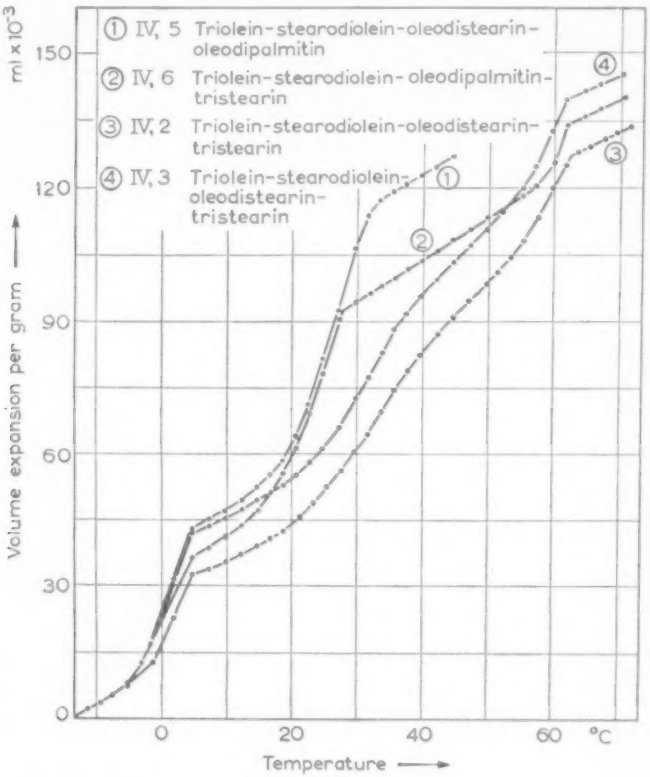


Fig. 10. Dilatometric curves for triglyceride mixtures.[19]
IV. (5) triolein 40·4%, stearodiolein 20·2%, oleodistearin 20·3%, oleodipalmitin 19·1%.
IV. (6) triolein 47·2%, stearodiolein 20·2%, oleodipalmitin 19·0%, tristearin 13·6%.
IV. (2) triolein 38·5%, stearodiolein 23·0%, oleodistearin 23·0%, tristearin 15·5%.
IV. (3) triolein 46·6%, stearodiolein 20·0%, oleodistearin 20·0%, tristearin 13·4%.

glycerides are separated by more than 15°C. Some further research was conducted on this particular aspect by one of the authors*, who found that the discrete melting segments illustrated by some of the examples cited above disappeared if the relative amounts of the components were altered. The discrete melting of triolein in the examples given above was found fortuitously because of the relatively large amount of this component in the various mixtures. The work on triglyceride mixtures pointed up the fact that unless one had an a

* LUNDBERG W. O. Private communication.

priori knowledge of the components in the mixture, it would be difficult if not impossible to ascribe any section of the dilatometric curve as being due to any particular glyceride. The work did reveal however that initial and final melting points could be determined and that the melting points of the more saturated glycerides were lowered by the presence of lower melting glycerides. This in part explains overlapping of melting sections. Indirect proof was obtained to show that solution of more saturated glycerides in the liquid glyceride did not occur. This is illustrated in Fig. 9 on curves 1 and 2. The final melting points of tripalmitin and tristearin were lowered from 69·9°C and 72·5°C to 57°C and 63°C respectively, and no apparent solution or melting has occurred between 5° and 40°C as determined by a comparison of measured and calculated volume change over this temperature interval, the latter indicated by the dotted line.

IX. Polymorphism

A number of investigations into polymorphic forms and transformations have been made on fatty materials. Dilatometry offers the obvious advantage of accurately measuring slow transformations at a selected temperature and allowing long periods of time to attain equilibrium.

A. Tristearin

Bailey and Singleton[12] reported dilatometric data on three polymorphic forms of tristearin including expansivities in the solid state and melting dilations which are given in Table 4. They found that transformation (indicated by volume contraction of form III began around 40° with rapid transformation at 48°. Transformation of form II likewise began at around 40° and rapid contraction was observed at 44°. The transformation did not proceed uniformly in either case, but showed plateaus. Both forms were transformed into form I and the dilatometric curves were blended to one curve at around 65°C. The course of the transformations were followed by taking X-ray photographs of the original samples and of portions of samples taken at intervals along the dilatometric curves.

Mahadevan and Lundberg[24] in a preliminary report on a study of the polymorphism of tristearin using volumetric dilatometers described the preparation of four apparently different solid forms that were obtained by different heating and cooling techniques. Heating curves for the several forms are shown in Fig. 11. It was not possible on the basis of the preliminary X-ray study to determine whether the four forms represented truly different polymorphic modifications.

Rapid cooling of the tristearin melt yielded a first form (gamma) which was the most unstable but which, nevertheless, because of the position of its heating curve in relation to the heating curve of stable liquid tristearin, was considered by them to possess crystallinity. Heating of the gamma form above 53°C resulted in a contraction until at 66° complete transition to a second and most stable (beta) of the four forms had been achieved.

A third form (alpha) was obtained when the tristearin melt was supercooled very slowly. At approximately 64° a marked contraction took place very rapidly followed by a partial re-expansion yielding a form whose density was less than that of either of the first two forms.

The alpha form exhibited a unique character that has not yet been explained. When gradually heated, contraction of this form began to take place at 64° and appeared to be complete at 71°. Cooling from this point gave a curve that coincided with that of the most stable or beta form. When the alpha form was heated above 71°, a melting dilation occurred which coincided with that of the

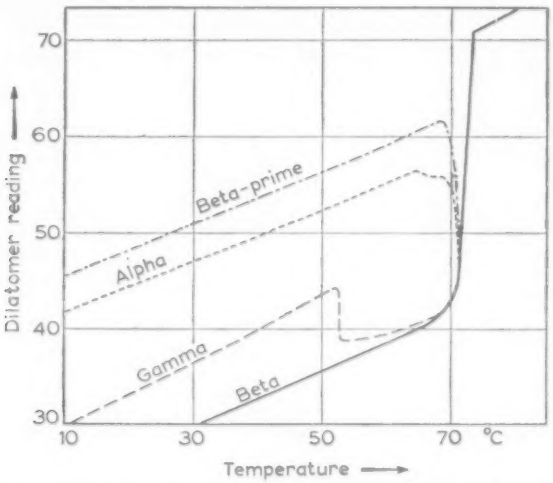


Fig. 11. Dilatometric curves for the polymorphic forms of tristearin.[24]

most stable form. Heating to 72·5° (very slightly below the melting point of 72·8°), and then cooling again gave a marked contraction until a temperature of approximately 71° was reached, at which point a very marked expansion occurred that was complete at approximately 68°. Thus there was obtained a fourth solid form (beta prime) with lower density than any of the other three forms. The densities and melting points obtained by MAHADEVAN and LUNDBERG[24] and by BAILEY and SINGLETON[12] are reported in Table 5.

Table 5. Densities of polymorphic forms of tristearin

Polymorphic form		*M.pt. °C*		*Density gm/ml*	
BAILEY and SINGLETON[12]	MAHADEVAN and LUNDBERG[24]				
Form III	Gamma	54·5	52·5	1·014 (− 38°)	0·9781 (25°)
Form II	Alpha	64·5	64·0	1·017 (− 38°)	0·9265 (25°)
	Beta-prime		70·0		0·9087 (25°)
Form I	Beta	72·5	72·8	1·043 (− 38·6°)	1·0125 (25°)

B. Monostearin

SINGLETON and VICKNAIR[23] studied the beta, beta prime, alpha, and subalpha forms of 1-monostearin with gravimetric dilatometers. The polymorphic forms were corroborated by X-ray analysis and confirmed the findings of LUTTON and JACKSON[25] who did not report dilatometric data in detail. Melting dilations and expansivities found by SINGLETON and VICKNAIR[23] are reported in Table 3, and the dilatometric data are presented graphically in Fig. 12. The beta form is the most dense in the crystalline state and the beta prime form is the least dense. The alpha form M.pt. 74·2°C was found to exist only when the subalpha form was warmed to 49°C, the subalpha form being obtained by rapid cooling

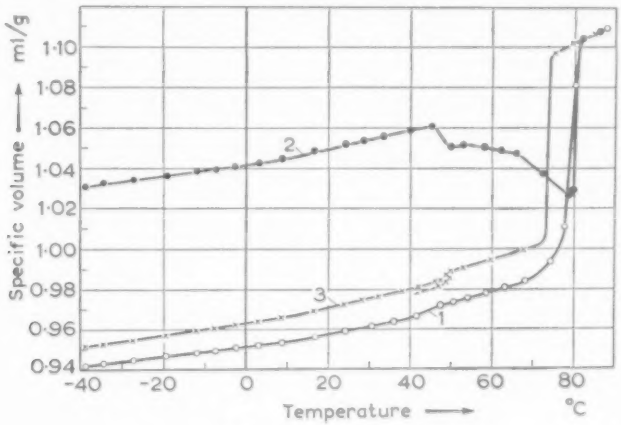


Fig. 12. Dilatometric curves for polymorphic forms of 1-monostearin,[23] 1-beta form, 2-beta prime form, 3-subalpha form.

of the melted sample. The transition of alpha and subalpha forms at 48–49°C was found to be reversible. The beta-prime form, obtained by fairly rapid crystallization from hexane, transformed to the stable modification before melting.

C. Stearic acid

SINGLETON, WARD, and DOLLEAR[16] measured melting dilations and expansivities for two polymorphic forms of stearic acid, and these data are reported in Table 1. They found that the *B* form changed into the more stable *C* form at temperatures considerably below the capillary M.pt. of 52·9°C. The transition could occur at 30–35°C, but required a period of 380 hr for complete transformation. This was confirmed by X-ray analysis, and this slow transformation without melting is a prime example of the usefulness of the dilatometer and indicates the unreliability of a visual method where the transformation must be sufficiently rapid to be observed. Volume contractions were observed in the solidus state for the *C* form which did not appear to be due to vacuoles or voids in the system.

D. Acetostearins

VICKNAIR, SINGLETON, and FEUGE[26] studied polymorphism in a new class of compounds with the dilatometer. They found three polymorphic forms in 1,2-diaceto-3-stearin and designated these as beta, alpha, and subalpha forms. The beta form obtained by crystallization from a solvent or the tempering of a previously solidified sample had a dilatometric M.pt. of 49·5°C. The alpha form was obtained by cooling a melted sample at a moderate rate. This form transformed to the stable beta form with or without melting depending on the temperature at which the sample was held. The subalpha form was found to exist only at temperatures below — 4°C and did not melt on raising the temperature, but did show some evidence of transformation to the alpha form between — 4°C and — 2°C. Three polymorphic forms beta prime, alpha, and subalpha were also found for 1-aceto-3-stearin. The subalpha form of this

Table 6. Melting dilations and expansivities of acetostearins[26]

Compound	*M.pt. °C*	*Melting dilation ml/gm*	*Expansivity ml/gm/°C*	
			Solid	*Liquid*
1,2-Diaceto-3-stearin:				
Beta form	49·5	0·1118	0·000301	0·00096
Alpha form	36·4	0·0649	0·00115	0·00096
1-Aceto-3-stearin:				
Beta prime	49·1	0·1185	0·000352	0·000945
Alpha prime	47·5	—	—	—

glyceride was found to be more stable than for the above-described glyceride. The melting point of the alpha form could not be determined dilatometrically because of an apparent transformation at a temperature below 47·5°C, the capillary melting point of this form. The subalpha form was obtained by cooling the melted acetostearin to below 0°C. Melting dilations and expansivities are given in Table 6.

E. Tung oil

SINGLETON and co-workers[27] studied polymorphism of alpha and beta tung oils, and the melting dilations and expansivities are reported in Table 7.

Three polymorphic forms, I, II, and III, were found for beta tung oil, and no polymorphism was found for the alpha tung oil. Form I was obtained by slow cooling of the sample or by tempering form II at 42–43°C. This transformation occurred without melting. Form III was obtained by rapid chilling in a mixture of acetone and dry ice. This form melted at 28°C, and form II was obtained by holding the sample at this temperature until solidification was completed. Transformation of forms II and III to form I were irreversible. These workers also studied the isomerization of alpha to beta tung oil in both light and dark under the influence of potassium iodide. After 3189 hr, equili-

brium was reached and a 4 per cent decrease in volume was observed for the sample exposed to light. The sample which was protected from light gave a slower reaction and had a 2·4 per cent decrease in volume in the same time.

Table 7. Melting dilations and expansivities of alpha and beta tung oils

Material	*M.pt. °C*	*Melting dilation ml/gm*	*Expansivity ml/gm/°C*	
			Solid	*Liquid*
Beta tung oil:				
Form I . . .	52·8[2]	0·078	0·00039	0·00087
Form II . . .	44·4[3]	0·067	0·00046	—
Form III . .	28·0[4]	0·061	0·00047	—
Alpha tung oil:				
	12·8	0·058	0·00030[1]	0·000815

[1] Observed expansivity 0·00059 but calorimetric data indicated melting started at − 43°C.
[2] Melting started at − 10°C.
[3] Transition started at 18°C.
[4] Transition started at − 10°C.

F. Cocoa butter

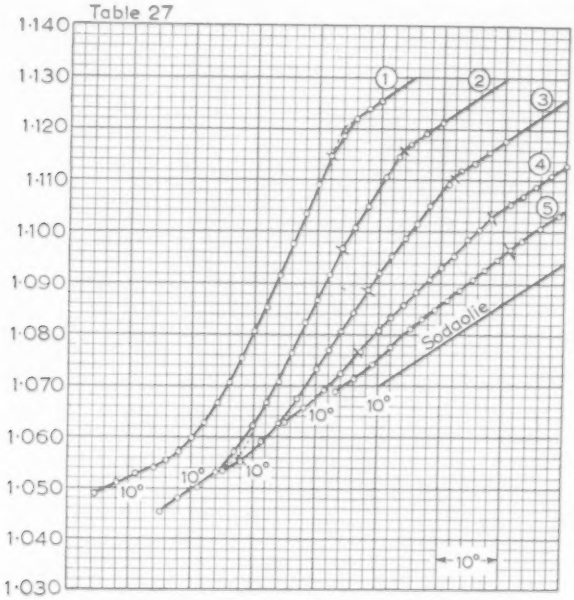
VAECK[20] studied polymorphism in cocoa butter using a volumetric dilatometer patterned after that used by HOFGAARD[7] but using a device like that used by BAILEY[8] for admitting mercury to the dilatometer bulb. VAECK found three polymorphic forms (beta, beta prime, and alpha) for cocoa butter, and reported specific volumes for each form. Difficulties were experienced in obtaining reliable measurements on the solid state for the beta form, primarily due to crevices or voids in the fat sample into which mercury could not penetrate. The difficulty was overcome by placing cocoa butter which was solidified in the stable form in the dilatometer bulb, filling the bulb with mercury, heating the sample and bulb to effect partial melting (31°C) and equilibration for one month each at 15°C and 7°C respectively. The dilatometric data on the beta form showed a transition around − 20°C, and the coefficient of expansion in the solid state was calculated over the temperature interval from − 20°C to 14°C. Heats of melting were calculated from the dilatometric data and discussed. The alpha form was found to transform to the beta-prime form at − 5°C.

X. NATURAL PRODUCTS

The greatest amount of published literature on dilatometric research has concerned natural fats and oils and hydrogenated products. HOFGAARD[7] made a detailed study of a number of vegetable oils, animal fats, and partially hydrogenated fats and oils. The study was extended to mixtures of the above and especially to mixtures of natural and hydrogenated products where the relative amounts were varied between wide limits. A volumetric type of dilatometer was used and measurements were made from 0°C to above the melting point of

the particular material. Most materials used by HOFGAARD[7] contained unsaturated acids and, since most measurements were made only down to 0°C, little data is available for calculation of the expansion in the solid state.

HOFGAARD found that the dilatometric curves for natural products were made up of linear segments which indicated discrete melting of glycerides or glycerides classes. These breakpoints or intersections were designated as K



1. Hærd. Sojaolie V
2. 75% hærd. Sojaolie V og 25% Sojaolie
3. 50% " " " " 50% "
4. 25% " " " " 75% "
5. 10% " " " " 90% "

Fig. 13. Dilatometric curves for hydrogenated soybean oil, soybean oil, and mixtures of soybean oil with hydrogenated soybean oil.[7]

(1) Hydrogenated soybean oil.
(2) 75% hydrogenated soybean oil + 25% soybean oil.
(3) 50% hydrogenated soybean oil + 50% soybean oil.
(4) 25% hydrogenated soybean oil + 75% soybean oil.
(5) 10% hydrogenated soybean oil + 90% soybean oil.

points with K_1 as the final melting temperature and K_2, K_3, etc., representing intersections at progressively lower temperatures. The linear segments were designated as K_1K_2, K_2K_3, etc. The use of the K points presents an interesting study.

An example of the existence of K points is shown in Fig. 13 where a series of dilatometric curves are presented for mixtures of soybean oil and soybean oil hydrogenated to an iodine value of 65·7 and where the ratios of the two components are varied. The curve representing the hydrogenated soybean oil does not

show any inflection points although this material would be expected to be composed of a number of glycerides. The addition of soybean oil brings out an inflection point, which shows a regular temperature dependence as the relative amount of soybean oil in the mixture is increased. The consistent lowering of the K_1 point (final melting point) as the proportion of liquid component is

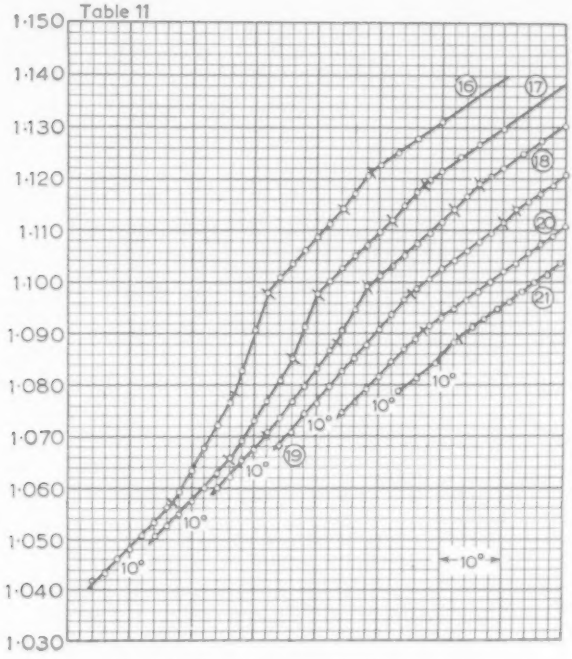


Fig. 14. Dilatometric curves for neutral lard, peanut oil, and mixtures of neutral lard and peanut oil.[7]

(1) Neutral lard.
(2) 75% lard and 25% peanut oil.
(3) 50% lard and 50% peanut oil.
(4) 25% lard and 75% peanut oil.
(5) 10% lard and 90% peanut oil.
(6) Peanut oil.

increased is readily understood, but the appearance and disappearance of the K_2 point is more difficult to understand. Fig. 14 shows a similar relation for mixtures of lard and peanut oil. The original lard shows five inflection points between 10° and 50°C and the temperatures of the inflection points decrease in a regular manner as the relative amount of peanut oil is increased. Some of the lower inflection points disappear completely. HOFGAARD studied a large

number of other mixtures and found in general that the K points varied in a regular manner as the relative proportions of components were varied. If the

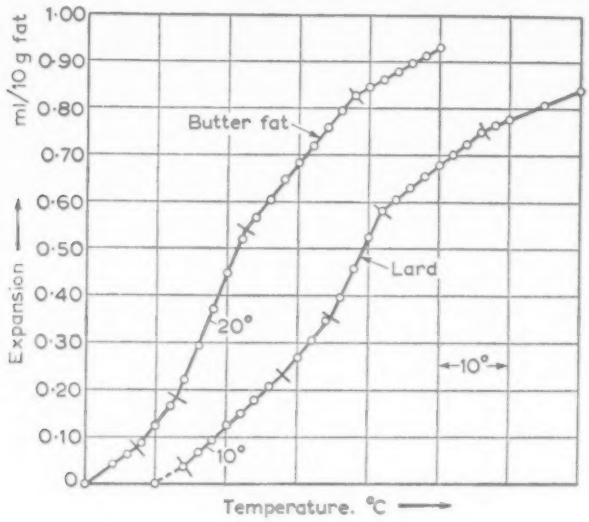


Fig. 15. Dilatometric curves for butterfat and lard.[28]

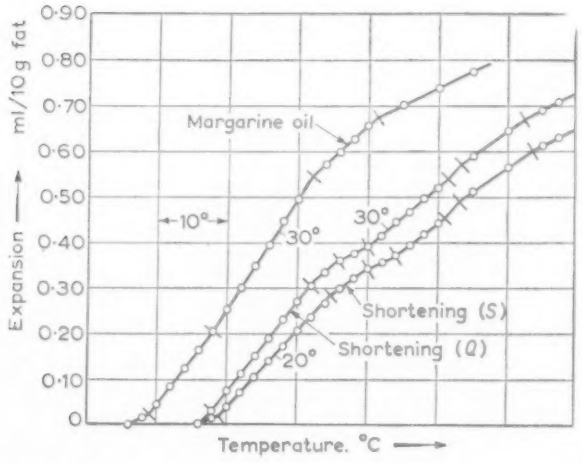


Fig. 16. Dilatometric curves for margarine, shortening chilled slowly (S), and shortening chilled quickly (Q).[28]

temperature for the inflection point is plotted against the proportion of one component a straight-line relationship is obtained.

Kraemer and Bailey[28, 29] have confirmed occurrence of inflection points in the dilatometric curves for butterfat, lard, margarine oil, and shortenings, as shown in Figs. 15 and 16. Four linear sections are seen in Fig. 15 for butterfat

and five sections for lard. The shortening illustrated in Fig. 16 shows seven linear sections indicating a more complex mixture of glycerides which might be anticipated due to the production of isoacids during hydrogenation.

It is difficult to assess the meaning of the appearance of linear sections in the natural products studied by HOFGAARD[7] and by BAILEY *et al.*[28] since the component glycerides in the mixtures were not known. At the outset it is difficult to reconcile the appearance of intercepts or K points, which would mean that a complex mixture of glycerides shows discrete melting, with the data reported by CRAIG *et al.*[19] who found that discrete melting was shown only in relatively simple mixtures where the melting points of the individual glycerides were at least 15° apart. However, the regular variation of K points with relative proportions of one component found by HOFGAARD may indicate that the appearance or disappearance of K points is more influenced by relative proportions of glycerides or glyceride classes than by separation of the melting points. It may well be a fortuitous circumstance that inflection points were not found in the work on synthetic glycerides due to the relative proportions of glycerides. Further research on synthetic mixtures would be required to properly assess the relative effects of proportions of glycerides as compared to the separation of melting points of components in the mixture.

XI. Determination of Solids Content

The principal interest in dilatometric research has been to assess the method for the determination of solid-liquid ratios in fats, oils, and derived products at various temperatures. It is obvious from the discussion of melting dilations of synthetic glycerides and natural and derived products that an accurate determination of solid content by using the dilatometric data as a phase diagram is very difficult. However, the use of some assumptions and approximations should yield reliable comparative data.

The volume-temperature relationship or dilatometric curve represents solid-liquid equilibria in the range from complete solidification to complete liquifaction, and hence could be used as a phase system. In such a system the simple ratio of the relative distances of a selected point from the liquidus and solidus lines should give the relative proportions of solid and liquid. This is true only if the following conditions are fulfilled:

(i) The melting dilations of all glycerides in the mixture are equal. It is obvious from earlier discussions that this condition is not fulfilled. The melting dilations vary from 0·08 ml/gm for triolein to 0·16 ml/gm for the saturated triglycerides with the values of the mixed glycerides of saturated and unsaturated acids falling between these values.

(ii) The dilatometric curve from the melting point of the first component in the mixture should be only melting of glycerides of higher melting points without discrete melting segments separated by linear sections representing the combined expansion of solid and liquid states. Fulfilment of this condition depends on the glyceride composition of the material being studied. If the

melting points of the components are sufficiently separated, linear expansion sections will be present.

It is rather obvious that an accurate determination of the proportion of solid material will depend on an a priori knowledge of the glycerides which are present, and mathematical adjustment to take care of the different melting dilations.[10, 19] For practical purposes, particularly in shortenings and margarines, certain assumptions may be made and reasonably accurate relative data may be obtained. In the first place principal interest is in the temperature region from 10–50°C, where the mono- and di-saturated glycerides melt. This would mean that if one is interested in solid content in the temperature region of 25°C one is dealing with melting of monosaturated glycerides and comparisons can be made of different mixtures. The same applies to measurements in the region of 35°C where the disaturated glycerides would be expected to show volume dilation due to melting. The fully saturated glycerides would be expected to show melting dilations at 50°C and above. The other variable in such determinations is the use of a base line which could be either the liquidus or solidus line. Due to the low melting points of the fully unsaturated glycerides the determination of the solidus line requires measurements at temperatures lower than — 30°C. The liquidus line is more easily and more accurately determined and there is no reason that this line cannot be used. The most general approach at present is to use assumed coefficients of expansion for liquid and solid states and an assumed specific volume and temperature for the solid state. The use of this technique will give relative data which can be compared.

Hofgaard[7] adopted the procedure of taking the specific volume of the solid material as 1·0100 for all samples and using a coefficient of 0·00050 ml/gm/°C and 0·00085 ml/gm/°C for the solidus and liquidus lines respectively. The solid content was then calculated as the ratio of the relative distances of the dilatometrically determined volume from the solidus and liquidus lines. Bailey and co-workers[28] used a liquidus coefficient of 0·00085 ml/gm/°C and a melting dilation figure of 0·1000 ml/gm. The solid content was calculated by measuring from the melting curve to the liquid line and taking each difference of 0·001 ml as corresponding to 1 per cent of solids. Fulton, Lutton, and Wille[9] describe in detail a dilatometric procedure for the determination of solid-content index. These workers use the figure 0·1000 for melting dilation and calculate the solid content index in the same manner as the simplified procedure suggested by Bailey. The general trend in calculation of solid content appears to be the use of 0·1000 ml/gm as the melting dilation. The temperature region 10–50°C would cover the range in which mono-, di- and tri-saturated triglycerides would melt. The melting dilations given in Table 4 for these triglycerides show variation from 0·1178 ml/gm for stearodiolein to 0·1671 ml/gm for tristearin. The glycerides containing isoacids would likely show melting dilations between these values, and the fully unsaturated glycerides would have melting dilations below 0·1000 ml/gm. The value 0·1000 ml/gm may be low if the content of fully unsaturated glycerides in

the commercial product is lower than 50 per cent. However, the figure 0·1000 as melting dilation is an arbitrary figure, and is a very convenient figure for calculating per cent of solids.

There is a need for a standard procedure which takes into account the size and shape of dilatometer bulb, size of sample, tempering procedure, and time to be allowed for equilibration. FULTON, LUTTON, and WILLE[9] have published a detailed procedure which takes all these variables into account.

XII. CONCLUSIONS

The dilatometer is a very accurate method of measuring small volume changes in a material which may have been caused by a change of temperature or by a phase transformation. The gravimetric type of dilatometer will detect smaller changes than a volumetric type, although the latter is preferred for rapid determinations. The dilatometer offers a distinct advantage over methods such as calorimetry in that much longer periods of time can be allowed for equilibrium.

More research is required on pure compounds to complete the data on expansivities, melting dilations, and the effect of substituents. More fundamental research is required on mixtures of pure compounds to evaluate the effect of one component on another. This type of research might well be extended to include solvents and a study of the effect of mutual solubilities. It is equally possible that the effect of tempering on crystal size and crystal growth could be studied by means of the dilatometer. In summation it might be said that only a beginning has been made in the use of this technique to investigate physical behaviour in fats and oils.

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9

ULTRA-VIOLET SPECTROPHOTOMETRY OF FATTY ACIDS

G. A. J. Pitt and R. A. Morton

CONTENTS

		Page
I.	INTRODUCTION	228
	A. Ultra-violet absorption	228
II.	ABSORPTION SPECTRA OF NATURALLY OCCURRING ACIDS	229
	A. Absorbing impurities in fatty acids	234
	B. Acetylenic acids	235
	C. Accuracy of spectroscopic analysis	238
III.	ALKALINE ISOMERIZATION OF UNCONJUGATED FATTY ACIDS	243
	A. Development of analytical method	246
	B. Time of heating	248
	C. Temperature	251
	D. Solvent for isomerization	251
	E. Calibration by means of experiments on purified acids	252
	F. Correction procedures	254
	G. Concentration of alkali	258
	H. Artifacts	268
	I. Adaptation to semi-micro and micro scale	270
	J. Standard methods	271
	K. Efficacy of the method	272
	REFERENCES	274

9

ULTRA-VIOLET SPECTROPHOTOMETRY OF FATTY ACIDS

*G. A. J. Pitt and R. A. Morton**

I. Introduction

The study of ultra-violet absorption spectra until comparatively recently required the use of photographic plates; this not only made the work tedious but also, because intensity determinations could only be made to ± 2 per cent, limited the analytical applications.

Growing appreciation of the value of the subject stimulated the replacement of spectrographic techniques by photoelectric spectrophotometric methods. Improvements in the design and production of instruments accelerated the switchover and brought many newcomers into the field. The result has been a prodigious increase in published work on ultra-violet absorption spectra; for example, more than a quarter of the experimental papers published in 1953 by the *Journal of the American Oil Chemists' Society* included spectroscopic data.

The present survey will be restricted in the first place to the spectra of the fatty acids (and of their isomers) which occur in natural triglycerides; and in the second place to the determination of polyunsaturated acids by the indirect method of alkali isomerization, which produces, in a controlled manner selectively absorbing acids.

A. Ultra-violet absorption

Using a quartz prism in air, the accessible ultra-violet region of the spectrum lies between 200 mμ and 400 mμ; the further ultra-violet region can be investigated with special apparatus. Most instruments in use today give reliable results above 220 mμ, and some are trustworthy to 210 or 200 mμ.

Ultra-violet absorption is associated with unsaturated *chromophores*, those with which we shall be concerned being the ethylenic, carboxylic, ketonic, and acetylenic groups. Alone or separated (insulated), these groupings do not give rise to appreciable absorption between 210 mμ and 400 mμ; but when conjugated they show intense selective absorption. (Many excellent reviews, e.g. Braude, 1945; Ferguson, 1948; and Gillam and Stern, 1954, discuss the relationship between structure and light absorption.) Among the fatty acids, therefore, those containing two or more conjugated double or triple bonds are of major spectroscopic interest.

In this review, we shall express the extinction of fatty acids in terms of

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ε—the molecular extinction coefficient—or of $E_{1\text{ cm}}^{1\text{ per cent}}$. Another very widely used measurement is the Specific Extinction (or Absorption) Coefficient: k or α. ε, $E_{1\text{ cm}}^{1\text{ per cent}}$, and k (or α) represent the extinction of a 1 cm layer of a molar, a 1 per cent solution and a 0·1 per cent solution respectively, i.e.

$$\frac{\varepsilon}{\text{mol. wt.}} = k \text{ or } \alpha = \frac{E_{1\text{ cm}}^{1\text{ per cent}}}{10}.$$

II. Absorption Spectra of Naturally Occurring Acids

By virtue of their carboxyl grouping, all fatty acids show absorption in the far ultra-violet. Rusoff, Platt, Klevens, and Burr (1945) found 'saturated' acids, e.g. myristic, to have a broad but weak absorption band ($\varepsilon \simeq 50$) with a maximum (λ_{max}) near 205 mμ; stronger absorption began at 185 mμ, rising steeply on the short wavelength side as far as the measurements extended. The weak band is intensified significantly in some branched chain fatty acids (Cason and Sumrell, 1951a), but the absorption of saturated fatty acids at wavelengths greater than 220 mμ can usually be neglected.

Monoethenoid acids, such as oleic, containing a carboxyl group and an ethylenic linkage distant therefrom, have λ_{max} about 183 mμ and show much more intense absorption. Polyunsaturated acids with insulated double bonds (i.e. separated from one another by at least one CH_2 group, e.g. linoleic, linolenic, arachidonic) absorb similarly in this region but more strongly. Usually, while the presence of two or more insulated chromophores has no effect on the position of λ_{max}, the intensity of absorption is additive. For the polyunsaturated fatty acids, Rusoff *et al.* (1945) found that the absorption around 190 mμ did not increase very greatly in arachidonic acid as compared with linoleic acid. Differences were more obvious between 210 mμ and 220 mμ; at 210 mμ the molecular extinction coefficient of arachidonic acid was about 80 times that of oleic acid. Barnes, Rusoff, Miller, and Burr (1945) and Rusoff *et al.* (1945) suggested that the extinction at 210 mμ was a measure of the number of double bonds present in a fatty acid or fat. Later work seems to show this is not so. Silk and Hahn (1954b) found that hexadecatetraenoic acid did not absorb as strongly as arachidonic acid, and Whitcutt and Sutton (1956) reported that eicosapentaenoic acid has the same absorption below 220 mμ as has arachidonic acid. Although the significance of absorption around 210 mμ is not clear, it can be said as a generalization that the presence of polyunsaturated acids in fats greatly increases the intensity of absorption around and just below 220 mμ without producing a maximum. In brief, above 220 mμ saturated fatty acids are practically transparent and unsaturated acids increase the unselective end absorption.

Of far greater spectroscopic interest are the *conjugated* polyene acids. With increasing conjugation of double bonds, λ_{max} moves towards the visible; Table 2 summarizes the spectroscopic characteristics of these acids. It will be seen that conjugated dienes have λ_{max} near 230 mμ, trienes near 270 mμ, and

tetraenes around 300 mμ; as conjugation increases, the extinction rises (see Fig. 1).

Fatty acids with five or more conjugated double bonds do not occur naturally but can be produced artificially from unconjugated isomers. The pentaene acids have λ_{max} near 330 mμ and the hexaenes near 350 mμ.

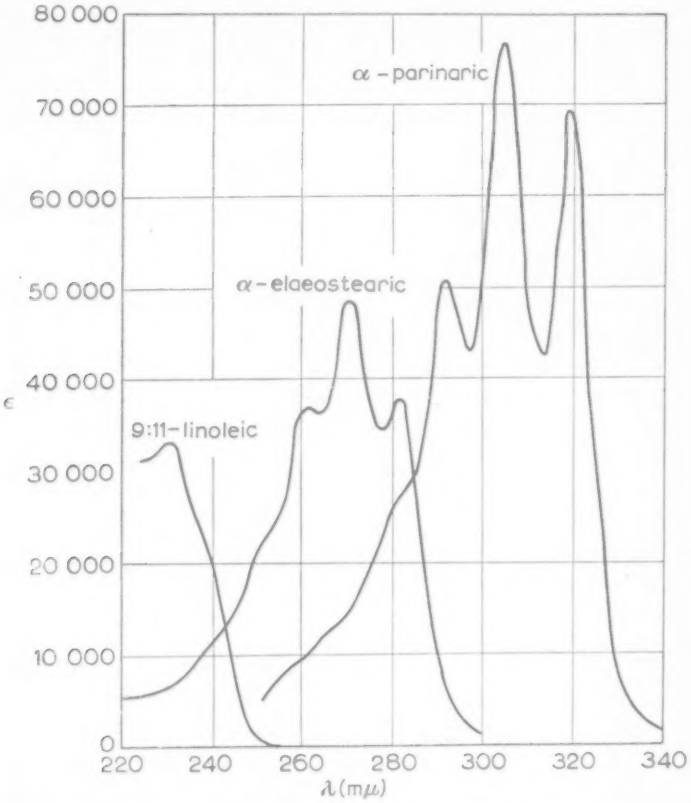

Fig. 1. Absorption spectra of trans-trans-9:11-linoleic, α-elaeostearic and α-parinaric acids. Solvent: ethanol.

(*By courtesy of the Research Association of British Paint, Colour, and Varnish Manufacturers.*)

The only dienoic acid isolated from a fat and shown spectroscopically to be conjugated is 9:14-dihydroxyoctadeca-10:12-dienoic acid from tung oil, and this may well be an oxidation artifact (Davis, Conroy, and Shakespeare, 1950). Other conjugated dienoic acids claimed to occur in nature include 12-methyloctadeca-9:11-dienoic (Hilditch, Meara, and Zaky, 1941), and octadeca-9:11-dienoic (Kartha and Menon, 1943). Of these, the second lacked spectroscopic verification, and the first is now accepted as erroneous, although there is still some disagreement about its true structure (Faure and Smith, 1956; Varma, Nath, and Aggarwal, 1956). It is true that certain fats always show a 230 mμ peak typical of conjugated dienoic acid, but this may be an

artifact; small amounts of conjugated acids can be produced from transparent precursors by relatively mild processing.

With the conjugated dienes should be considered the monoenoic acids having their ethylenic link conjugated with the carboxyl group. A number of such acids, both naturally occurring and synthetic, have received much attention in recent years. Most of them do not come into the restricted scope of this review, but mention must be made of the α-ethylenic acids found in the tubercle bacillus. None of these has been unequivocally identified, but it seems probable that they include mycolipenic acid (Bailey, Polgar, and Robinson, 1953). The subject is still controversial (Cason and Fonken, 1956) but mycolipenic acid has been synthesized and examined spectroscopically (Bailey *et al.* 1953). α-Ethylenic acids usually show λ_{max} around 208–210 mμ and a lower ε_{max} than do the conjugated dienes, as the carboxyl group is a less effective chromophore than a second ethylenic link. In mycolipenic acid, the introduction of a methyl group on one side of the ethylenic link shifts λ_{max} to slightly longer wavelengths (Cason and Sumrell, 1951b; Cason, Allinger, and Williams, 1953). Acids of this type with branched chains are spectroscopically anomalous. The nature of the branching affects both the position of λ_{max} and also ε_{max} (Braude and Evans, 1955) but it may affect the free acid differently from its ester (Cason, Allinger, and Sumrell, 1953; Cason and Rinehart, 1955). These acids do not show the usual solvent shift: they do not obey the general rule that polar solvents tend to move λ_{max} to longer wavelengths and depress the maximum extinction (Cason, Allinger, and Allen, 1953; Cason and Fonken 1956).

It is possible that some of these apparent anomalies may be related in part to the observation that the anions of acids having a carboxyl group conjugated to an unsaturated system have spectra different from those of the uncharged molecules. Full details of the shorter chain acids are given by Allan, Jones, and Whiting (1955). The phenomenon is much more marked in acids with polyene chains such as those related to vitamin A (Robeson, Cawley, Weisler, Stern, Eddinger, and Chechak, 1955; Huisman, Smit, van Leeuwen, and van Rij, 1956) and with longer conjugated systems such as β-14′-apo-carotenoic acid (Fazakerley, 1957). The unusual solvent shift of λ_{max} in deca-trans-2:trans-4-dienoic acid reported by Jacobson (1953) may be due to this effect.

Deca-2:4-dienoic (stillingic) acid with its two ethylenic links conjugated to the carboxyl group shows λ_{max} near 260 mμ with an extinction coefficient lower than that of the conjugated trienoic acids such as elaeostearic (see Table 2). Although ε_{max} of the methyl ester is the same as that of the acid, λ_{max} is slightly different (Crossley and Hilditch, 1949, 1950).

The next higher homologue, dodeca-2:4-dienoic acid, has been reported in the seed oil of *Sebastiana lingustrina* by Holman and Hanks (1955). The position of maximum absorption is similar, but they found the molecular extinction coefficient to be considerably higher than those determined for stillingic acid and all synthetic α-γ-dienoic acids by other workers (Crossley and Hilditch, 1949, 1950; Devine, 1950; Crombie, 1955; Crombie and Manzoor-

I-KHUDA, 1956; see also references in ALLAN, JONES, and WHITING, 1955). Although these acids are not easy to handle there seems a discrepancy to be explained.

The absorption curves of these acids differ markedly in shape from those of the conjugated triethenoid acids in that they do not show fine structure (see Fig. 2). The absorption spectra of fatty acids with three or more conjugated double bonds have three peaks: a main one, with a subsidiary maximum on either

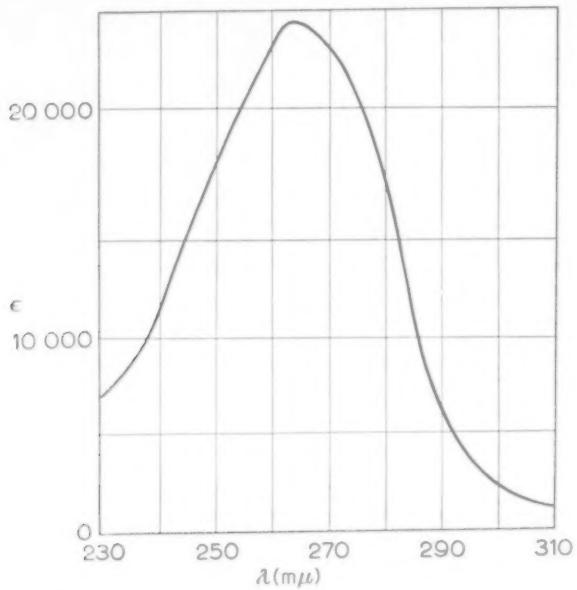

Fig. 2. Absorption spectrum of methyl deca-2:4-dienoate. Solvent: ethanol. (CROSSLEY and HILDITCH, 1949.)

side. The absorption of at any rate the trans-trans conjugated dienes is probably fundamentally similar. KASS (1944) gives a curve for conjugated linoleic acid (reproduced in MARKLEY, 1947) showing three maxima and indications of fine structure have been reported by other workers (NICHOLS, HERB and RIEMENSCHNEIDER, 1951; SCHMID and LEHMANN, 1950; GUNSTONE and RUSSELL, 1955). In cis isomers, this is less likely to be observed (see below). As many curves not showing fine structure have been published for trans-trans conjugated dienoic acids, it seems probable either that the resolution was intrinsically less marked or that the specimens contained some cis isomers. On the other hand unreal 'maxima' have been obtained when a compound with a flat maximum is studied by the photographic method, e.g. sorbic acid (EISNER, ELVIDGE, and LINSTEAD, 1953). Braude attributed the spurious peaks to an optical illusion in reading the plate (ALLAN *et al.* 1955).

With longer chains of conjugated double bonds, the peaks are spread further apart. HAMMOND and LUNDBERG (1953) have shown that the maximum of

each of the three peaks can be predicted from the relationship: $\lambda^2 = A + B_n$, where A and B are constants and n is the number of conjugated double bonds. A is the same for all peaks of all acids; B represents one of three constants B_1, B_2, or B_3, applicable to the maxima, the longer wavelength subsidiary peaks, and the shorter wavelength subsidiary peaks respectively. B_1, B_2, and B_3 differ in such a way that higher values of n cause the three peaks to spread out.

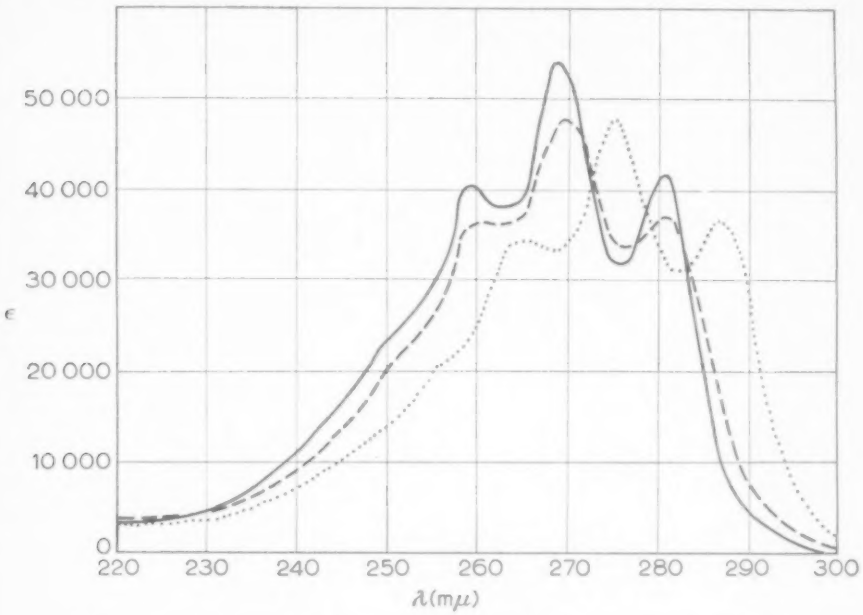

Fig. 3. Absorption spectra of geometrical isomers of octadeca-9:11:13-trienoic acid. α-elaeostearic, – – – –; β-elaeostearic ———; and punicic acid . . . . . Solvent: cyclohexane.

(*By courtesy of the Research Association of British Paint, Colour, and Varnish Manufacturers.*)

However, it is obvious from the equation that with increasing values of n, the intervals between the main peaks of successive member of the series will become smaller. As a result, the absorption curves of the higher polyene acids overlap; to determine them in mixtures simultaneous equations must be set up.

For all substances with polyene chromophores, the geometrical isomers differ in respect of absorption spectra. In the fatty acids, the all-trans form has the highest extinction coefficient. Cis-isomers not only have less intense absorption, but usually also have their maxima displaced to longer wavelengths and vibrational structure is not so clearly seen, i.e. the secondary peaks are less sharp. Fig. 3 shows the differences between the three isomeric octadeca-9:11:13-trienoic acids: α-elaeostearic (cis-trans-trans), β-elaeostearic (trans-trans-trans), and punicic (cis-cis-trans) acids.

Most naturally occurring conjugated acids contain at least one cis linkage in their chromophores; but many agents will cause them to isomerize, in part

at least, to their all-trans isomer. Consequently many specimens of conjugated acids isolated from natural sources contain varying proportions of isomers, which complicate analyses. Equations based on differences in extinction coefficients allow the proportions of different isomers to be estimated, provided the absorption spectra of the components show enough differences. O'Connor, Heinzelman, McKinney, and Pack (1947), O'Connor, Heinzelman, Pack, and Planck (1953), and May (1952) have devised such procedures for the mixtures of α-elaeostearic acid (naturally occurring) and β-elaeostearic acid (artifact) found in tung oil.

The accuracy of these methods is open to some doubt, largely owing to the difficulty of preparing pure standard specimens uncontaminated with other isomers. Paschke and Wheeler (1955) obtained impossible results with their elaeostearic preparations when using the procedure of O'Connor *et al.* (1947). Such interlaboratory differences probably stem in part from the need to measure points on steep extinction curves. In such circumstances small instrumental deviations have large consequences.

Difficulties from cis-trans isomerism arise also in the spectrophotometric determination of the conjugated diene acids produced from ricinoleic acid in dehydrated castor oil. This complex problem is discussed by Herb (1955). Current procedures are almost certainly somewhat inaccurate and there seems no immediate prospect of substantial improvement.

A. Absorbing impurities in fatty acids

The best known polyunsaturated fatty acids, such as linoleic and linolenic, have their double bonds insulated and so do not, when pure, exhibit selective absorption in the more accessible region of the spectrum, but only 'end' absorption, i.e. the 'skirt' of the absorption band which goes well below 200 mμ. In practice, solutions of such acids tend to show peaks or inflexions above 220 mμ; these arise from impurities and their intensities are a useful guide to the degree of purity of a product. For example, Fig. 4 shows the absorption curves of linolenic-acid specimens prepared by O'Connor, Heinzelman, Caravella, and Bauer (1946). Preparation *A* was made using the normal low-pressure distillation without taking stringent precautions against oxidation; preparation *B* was a molecular distillate from which oxygen had been excluded. *A* shows obvious signs of conjugated tri- and tetra-enes, whereas *B* does not.

Similar spurious effects can be produced by oxidation, by various metals and other catalysts, by distillation and by most manipulations. Sometimes the chromophore arises from the migration of the ethylenic linkages to form a conjugated system (as in alkali isomerization), but this is not the only mechanism, other chromophores (some of uncertain constitution) being formed. Undoubtedly the most common cause of artifacts is oxidation; by and large, the more oxidized a fat is, the stronger its ultra-violet absorption. (This, of course, does not apply to the relatively uncommon fats which naturally contain conjugated double bonds; here oxidation causes destruction of the chromophore.) In addition to compounds absorbing selectively in the ultra-violet, oxidation

produces other substances which, although they do not themselves show absorption bands, readily give ultra-violet chromophores on simple treatment such as heating (SWAIN and BRICE, 1949) or bleaching with fuller's earth (MITCHELL and KRAYBILL, 1942).

Oxidation products bedevil ultra-violet spectrophotometry of fats by producing artifacts and by masking absorption of analytical interest where only small amounts of conjugated substances are being investigated. These oxidation products are numerous and of somewhat uncertain structure. The autoxidation of fats has been reviewed in an earlier volume of this series by HOLMAN (1954), who gave references to spectroscopic work.

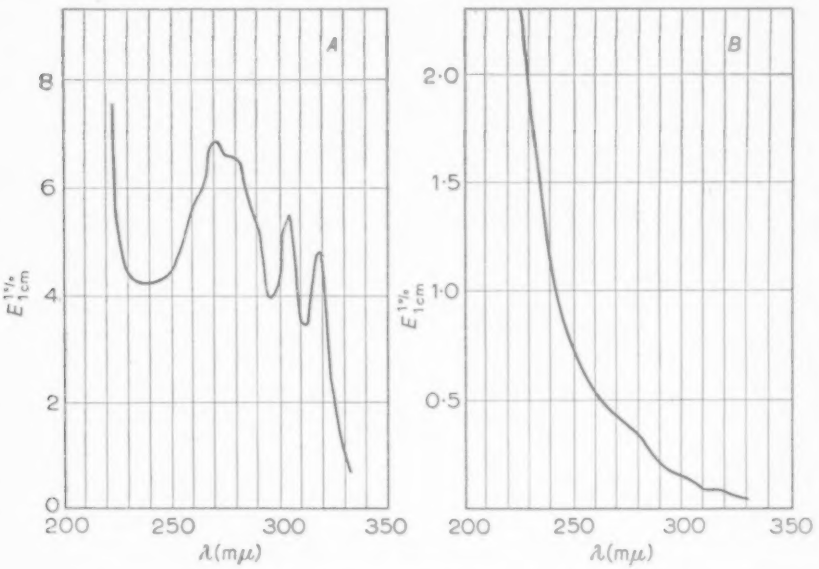

Fig. 4. Absorption spectra of specimens of linolenic acid. *A*: prepared by routine procedure; *B*: oxygen carefully excluded. Solvent: cyclohexane. (O'CONNOR *et al.*, 1946.)

B. Acetylenic acids

The first acetylenic acid to be found in fats was tariric (octadec-6-ynoic) acid; since then others have been isolated. Selective absorption appears in the accessible ultra-violet only when the acetylenic link is conjugated with a double bond or another triple bond. Acids containing these chromophores have recently been studied: ximenynic (santalbic), hydroxyximenynic (hydroxysantalbic), erythrogenic (isanic), isanolic, and bolekic acids. Their spectroscopic characteristics are summarized in Table 2.

Erythrogenic (isanic) acid—octadec-17-ene-9:11-diynoic—occurs in boleka nuts (*Onguekoe gore* Engler). The insulated ethylenic group, of course, makes no appreciable contribution to the observed extinction coefficient, but the conjugated acetylenic links give rise to three characteristic sharp bands of low

intensity between 227 and 254 mμ. These are most clearly seen in the synthetic acid (BLACK and WEEDON, 1953)—see Fig. 5.

The crude natural acid has a more complex spectrum; it has other bands at longer wavelengths, which are due to an enediyne contaminant. These appear in all specimens of natural erythrogenic acid which have so far been reported—see, for instance, CASTILLE (1940), BLACK and WEEDON (1953), and SEHER

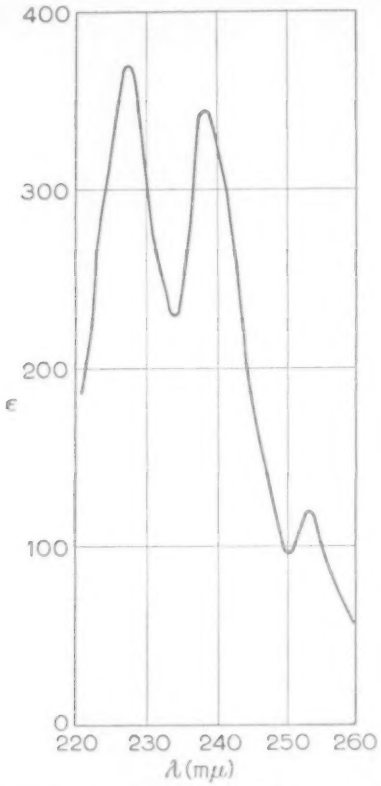

Fig. 5. Absorption spectrum of erythrogenic (isanic) acid. Solvent: ethanol. (WEEDON, private communication.)

(1954)—but BLACK and WEEDON have shown that it can be eliminated by preparing the ethanolamide of erythrogenic acid, which is much more easily purified than is the free acid. Table 1 shows the ultra-violet absorption of synthetic erythrogenic acid and of its ethanolamide, compared with that of the purified ethanolamide of natural erythrogenic acid, which is very similar to that of the synthetic compound. In the same table are given the figures for the natural free acid which contains an enediyne impurity. This contaminant has been isolated by MEADE, and named by him bolekic acid (MEADE, private communication)—see Fig. 6.

SEHER (1954) has isolated from boleka oil another enediynoic acid, which

appears identical with the isanolic acid reported by KAUFMANN, BALTES, and HERMINGHAUS (1951); he has produced evidence that it is 8-hydroxy octadec-14-ene-10:12-diynoic acid.

Tables 1 and 2 show the very low intensity of diyne absorption relative to that of diene or even of enediyne chromophores. In samples of erythrogenic acid containing only small amounts of bolekic acid, the absorption of the impurity may be greater than that from erythrogenic acid itself. Workers have

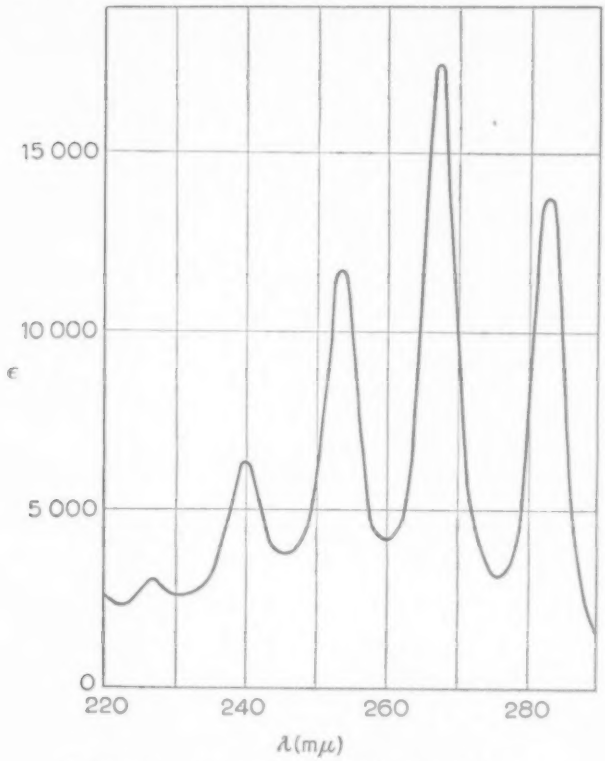

Fig. 6. Absorption spectrum of bolekic acid. Solvent: ethanol. (MEADE, private communication.)

been misled by this: KAUFMANN *et al.* (1951) identified isanolic acid as a hydroxy derivative of erythrogenic (isanic) acid, from the strong resemblance of the absorption spectra of the two acids. Being wise after the event, we can now recognize that the absorption curves shown by KAUFMANN *et al.* for erythrogenic acid are those given by the enediyne chromophore of an impurity.

Another acetylenic acid has been isolated from the kernel oils of several of the *Santalales*: octadec-11-en-9-ynoic acid (LIGTHELM, SCHWARTZ, and von HOLDT, 1952). It has λ_{max} 229 mμ with ε_{max} 16,200, and a slight inflexion at 240 mμ (ε about 12,000), i.e. absorption characteristic of a conjugated enyne—similar to a diene but of lower intensity. LIGTHELM *et al.* named it ximenynic acid as they

first isolated it from *Ximenia* species but GUNSTONE and MCGEE (1954), GRIGOR, MACINNES, MCLEAN, and HOGG (1955) and GUNSTONE and RUSSELL (1955) have shown that it is identical with santalbic acid, which had previously been isolated by MADHURANATH and MANJUNATH (1938)—see Table 2. LIGTHELM (1954) has also obtained from *Ximenia caffra* Sond. oil a derived hydroxy acid, 8-hydroxyoctadec-11-en-9-ynoic, which can be called either hydroxyximenynic or hydroxysantalbic acid.

In recent years, numerous naturally occurring acetylenic acids and their derivatives have been isolated from a variety of sources, e.g. essential oils and

Table 1. Absorption peaks of erythrogenic acid and related substances

Solvent: ethanol; λ in mμ (ε given in brackets below) (compiled from BLACK and WEEDON, 1953, and private communications from Dr. E. M. MEADE and Dr. B. C. L. WEEDON)

Compound	$\lambda\lambda_{max}$				
Synthetic erythrogenic acid . .	227 (370)	238 (344)	253 (120)		
Ethanolamide of synthetic erythrogenic acid	227 (640)	238 (444)	251 (181)		
Ethanolamide of natural erythrogenic acid	227 (650)	238 (438)	251 (228)		
Natural erythrogenic acid . . .	227 (480)	240 (480)	255 (384)	267 (300)	280 (260)
Bolekic acid	227 (3060)	240 (6300)	253·5 (11,700)	267 (17,400)	282·5 (13,600)

the culture liquids of fungi. Although they are of great spectroscopic interest, these acids are not known to occur in triglycerides and will not be considered further.

C. Accuracy of spectroscopic analysis

The extinction values given for the acids in Table 2 include the highest recorded for any one solvent. In addition, commonly used values have been given, although they are not the highest reported. (For instance, it is noticeable that extinctions of conjugated dienoic acids determined by photographic methods appear, possibly fortuitously, to run higher than those found photoelectrically.) In order to display solvent effects, values have been tabulated for impure specimens of some acids, which happen to have been studied in various solvents. Further, some apparently low values have been included because they are accompanied by more details of the subsidiary peaks than are the reports on purer specimens.

Even when these additions are allowed for, a critical scrutiny of Table 2 will show discordant values for specimens which on chemical evidence appear to be equally pure. The discrepancies should not be ignored but there is not enough

Table 2. Ultra-violet absorption data for fatty acids

Acid	*Configuration*	*Trivial name*	*Solvent*	λ_{max} (mμ)	ϵ_{max}	$E^{1\ per\ cent}_{1\ cm}$	*Reference*
Conjugated dienoic							
Octadeca-9:11-dienoic	trans-trans	9:11-linoleic	petrol	231	35,000 (*p*) (*c*)	1,250 (*p*) (*c*)	VAN DER HULST (1935)
,,	,,	(MANGOLD'S acid)	,,	231·5	33,300 (*p*)	1,190 (*p*)	KAUFMANN *et al.* (1950)
,,	,,	,,	ethanol	231	33,300	1,190	GUNSTONE and RUSSELL (1955)
,,	,,	,,	petrol	232	32,200	1,150	KASS (1944)
,,	,,	,,	isooctane	231	31,900	1,140	WITNAUER *et al.* (1949)
,,	cis-trans	9:11-linoleic	ethanol	234	24,600	878	AHLERS *et al.* (1953)
Octadeca-10:12-dienoic	trans-trans	10:12-linoleic	neo-hexane	231	33,300	1,190	BRICE and SWAIN (1945)
,,	,,	(VON MIKUSCH'S acid)	ethanol	231	32,800	1,170	,, ,, ,,
,,	,,	,,	petrol	232	32,200	1,150	KASS (1944)
,,	,,	,,	isooctane	231	31,700	1,130	WITNAUER *et al.* (1949)
,,	trans-cis	10:12-linoleic	—	232 (*m*)	28,700 (*m*)	977 (*m*)	JACKSON *et al.* (1952)
,,	cis-cis	,,	methanol	235 (*m*)	27,900 (*m*)	950 (*m*)	ALLEN (1956)
9:14-dihydroxyoctadeca-10:12-dienoic	—	—	ethanol	229	39,260	1,260	DAVIS *et al.* (1950)
Conjugated trienoic							
Octadeca-9:11:13-trienoic	cis-trans-trans	α-elaeostearic	methanol	261 (*c*) 271 (*c*) 282 (*c*)	38,900 (*c*) 52,800 (*c*) 41,200 (*c*)	1,400 (*c*) 1,900 (*c*) 1,480 (*c*)	O'CONNOR *et al.* (1953)
,,	,,	,,	petrol	270·5	52,600	1,890	KASS (1944)
,,	,,	,,	ethanol	270	51,000	1,834	O'CONNOR *et al.* (1947)
,,	,,	,,	isooctane	260 (*c*) 270 282 (*c*)	35,900 (*c*) 47,300 36,400 (*c*)	1,290 (*c*) 1,700 1,310 (*c*)	O'CONNOR *et al.* (1945) — These values refer to same sample
,,	,,	,,	ethanol	261 271 281	36,000 47,000 38,000	1,290 1,690 1,370	CROMBIE and TAYLER (1954)
,,	,,	,,	cyclo-hexane	262 271·5 283	36,200 48,100 37,000	1,300 1,730 1,330	MAY (1952)
,,	,,	,,	cyclo-hexane	271·5	49,600	1,783	PASCHKE and WHEELER (1955)

Notes: (*a*) determined on methyl ester of acetate.
(*c*) calculated from published graphical data.
(*m*) determined on methyl ester.
(*p*) determined photographically.
(*q*) possibly the same as mycolipenic acid (polemical), see CASON and FONKEN (1956).

Table 2—(contd.)

Acid	*Configuration*	*Trivial name*	*Solvent*	λ_{max} (mμ)	ϵ_{max}	$E^{1\ per\ cent}_{1\ cm}$	*Reference*
Octadeca-9:11:13-trienoic	trans-trans-trans	β-elaeostearic	ethanol	259 268 279	47,000 61,000 49,000	1,690 2,190 1,760	Crombie and Tayler (1954)
,,	,,	,,	methanol	258 (*c*) 268 (*c*) 279 (*c*)	45,100 (*c*) 61,200 (*c*) 47,300 (*c*)	1,620 (*c*) 2,200 (*c*) 1,700 (*c*)	O'Connor *et al.* (1953)
,,	,,	,,	cyclo-hexane	259 (*c*) 269 280 (*c*)	40,900 (*c*) 56,200 42,800 (*c*)	1,470 (*c*) 2,024 1,540 (*c*)	O'Connor *et al.* (1947) — values on same specimen
,,	,,	,,	ethanol	268	60,100	2,161	O'Connor *et al.* (1947) — same specimen
,,	,,	,,	petrol	268	59,800	2,150	Kass (1944)
,,	,,	,,	neo-hexane	267	59,500	2,140	Brice and Swain (1945) — same specimen
,,	,,	,,	ethanol	268	58,100	2,090	,, ,, ,, — same specimen
,,	,,	,,	cyclo-hexane	259 269 280	40,300 54,900 41,700	1,450 1,975 1,500	May (1952)
,,	cis-cis-trans	punicic	cyclo-hexane	265·5 275 287	— 47,800 —	— 1,719 —	Ahlers *et al.* (1954)
Octadeca-10:12:14-trienoic	trans-trans-trans	γ-elaeostearic	petrol	268	52,480	1,886	Kaufmann *et al.* (1950)
,,	,,	,,	,,	268	60,100	2,161	Kass (1944)
18-hydroxyoctadeca-9:11:13-trienoic	cis-trans-trans	α-kamlolenic	ethanol	262 (*c*) 270 282 (*c*)	39,400 (*c*) 52,900 41,500 (*c*)	1,340 (*c*) 1,800 1,410 (*c*)	Gupta *et al.* (1953)
,,	,,	,,	,,	261 271 282	40,500 52,000 42,000	1,380 1,770 1,430	Crombie and Tayler (1954)
,,	trans-trans-trans	β-kamlolenic	,,	259 (*c*) 268 280 (*c*)	44,400 (*c*) 58,500 45,600 (*c*)	1,510 (*c*) 1,990 1,550 (*c*)	Gupta *et al.* (1953)

Table 2—(contd.)

Acid	*Configuration*	*Trivial name*	*Solvent*	λ_{max} (mμ)	ε_{max}	$E^{1\ per\ cent}_{1\ cm}$	*Reference*
18-hydroxyoctadeca-9:11:13-trienoic	trans-trans-trans	β-kamlolenic	ethanol	258 268 279	45,000 60,000 47,000	1,530 2,040 1,600	Crombie and Tayler (1954)
4-oxo-octadeca-9:11:13-trienoic	cis-trans-trans	α-licanic	petrol	272	50,200	1,720	Kaufmann *et al.* (1950)
,,	trans-trans-trans	β-licanic	,,	268	56,400	1,930	,, ,, ,,
Conjugated tetraenoic							
Octadeca-9:11:13:15-tetraenoic	2 or 3 trans, 2 or 1 cis	α-parinaric	heptane	292·5 306·5 321·5	52,700 77,900 66,300	1,910 2,820 2,400	Kaufmann and Keller (1948)
,,	2 or 3 trans, 2 or 1 cis	,,	cyclo-hexane	293 305 319	50,300 77,300 69,100	1,820 2,800 2,500	Winter *et al.* (1948)
,,	2 or 3 trans, 2 or 1 cis	,,	ethanol	292 305 319	50,500 76,700 69,200	1,840 2,780 2,520	Ahlers *et al.* (1953)
,,	trans-trans-trans-trans	β-parinaric	heptane	288 301·5 315·5	56,400 87,100 77,900	2,040 3,150 2,820	Kaufmann and Keller (1948)
,,	trans-trans-trans-trans	,,	ethanol	288 301 316	— 81,700 —	— 2,960 —	Ahlers *et al.* (1953)
Eicosa-?:?:?:?:?-tetraenoic	—	alkali isomerized arachidonic	,,	286 300 315	57,800 73,000 57,800	1,900 2,400 1,900	Mowry *et al.* (1942)
Conjugated pentaenoic							
Eicosa-?:?:?:?:?:?-pentaenoic	—	alkali isomerized eicosapentaenoic	methanol	315 328 346	not pure specimen	— — —	Herb *et al.* (1951)
Conjugated hexaenoic							
Docosa-?:?:?:?:?:?:?-hexaenoic	—	alkali isomerized docosahexaenoic	,,	333 353 374	not pure specimen	— — —	Hammond and Lundberg (1953)

Table 2—(contd.)

Acid	*Configuration*	*Trivial name*	*Solvent*	λ_{max} (mμ)	ε_{max}	$E^{1\ per\ cent}_{1\ cm}$	*Reference*
Monoenoic conjugated with carboxyl group							
2:4:6-trimethyltetracos-2-enoic	—	mycolipenic	methanol	214	13,200	323	Bailey *et al.* (1953)
2:4:?-trimethyltetracos-2-enoic (*q*)	trans	C_{27}-phthienoic	alcohol	216	11,140	279	Cason and Sumrell (1951b)
,,	,,	,,	heptane	218	13,200	323	Allen and Cason (1956)
Dienoic conjugated with carboxyl group							
Deca-2:4-dienoic	trans-cis	stillingic	cyclo-hexane	260 (*m*)	25,800 (*m*)	1,420 (*m*)	Devine (1950)
,,	,,	,,	ethanol	260	24,000	1,427	Crossley and Hilditch (1950)
,,	,,	,,	,,	264 (*m*)	24,000 (*m*)	1,317 (*m*)	Crossley and Hilditch (1949)
Dodeca-2:4-dienoic	,,	—	methanol	260	33,400	1,700	Holman and Hanks (1955)
Conjugated enynoic							
Octadec-11-en-9-ynoic	trans	ximenynic (natural)	cyclo-hexane	229	16,200	583	Ligthelm *et al.* (1952)
,,	,,	ximenynic (synthetic)	ethanol	229	16,610	597	Grigor *et al.* (1954)
,,	,,	santalbic (natural)	,,	228	16,600	597	Gunstone and McGee (1954)
8-hydroxyoctadec-11-en-9-ynoic	,,	hydroxyximenynic or hydroxysantalbic	cyclo-hexane	230 (*a*)	16,100 (*a*)	460 (*a*)	Ligthelm (1954)
Conjugated diynoic							
Octadec-17-ene-9:11-diynoic	—	erythrogenic (isanic)	ethanol	227 238 253	370 344 120	13·5 12·5 4·4	Black and Weedon (1953); Weedon (private communication)
Conjugated enediynoic							
Octadec-?-ene-?:?-diynoic	cis	bolekic	,,	227 240 253·5 267 282·5	3,060 6,300 11,700 17,400 13,600	112 230 427 635 496	Meade (private communication)
8-hydroxyoctadec-14-ene-10:12-diynoic	—	isanolic	—	230 (*c*) 240 (*c*) 252·5 (*c*) 265 (*c*) 279 (*c*)	6,120 (*c*) 6,250 (*c*) 6,380 (*c*) 8,240 (*c*) 6,520 (*c*)	211 (*c*) 216 (*c*) 220 (*c*) 284 (*c*) 225 (*c*)	Seher (1954)

evidence to settle the most accurate values. It is commonly assumed that the higher the observed extinction coefficient obtained, the purer the sample of acid. This is not always justifiable; an obvious exception is erythrogenic acid (see Table 1). Also, data for cis-acids must be scrutinized carefully; a mixture of a cis-acid with some of its trans-isomer will absorb more strongly than will a pure preparation of the cis-acid.

These discrepancies in Table 2 emphasize that the degree of purity needed for spectroscopic investigations on unsaturated acids is not easy to attain. There are instrumental difficulties too. With the narrow peaks found in some of these acids, the extinction values obtained are somewhat dependent on the performance of the instrument; unless narrow slits are used, low readings will be recorded. (An extreme example of this is found in measuring the 374 mμ peak of conjugated hexaene acids—see p. 267 and Fig. 17.) The importance of the slit width has been discussed recently by Herb (1955) who mentions discrepancies that may be introduced through lack of attention to this variable. Instrumental errors are also discussed by Goldring, Hawes, Hare, Beckman, and Stickney (1953).

Although spectrophotometric techniques have made possible great analytical advances, the absolute accuracy attainable has sometimes been overestimated. In the field of fatty-acid analysis, the complexity of the problems makes it wise not to push calculations too far.

III. Alkaline Isomerization of Unconjugated Fatty Acids

The commonest polyunsaturated fatty acids have their double bonds insulated and therefore do not show absorption peaks in the ultra-violet. If the double bonds can be rearranged so as to form a conjugated system, selective absorption will appear in the ultra-violet region. This change is the basis of the most widely used application of spectroscopy to fat chemistry: polyunsaturated acids are converted into conjugated isomers by heating in alkali and the intensity of the resulting selective absorption is used as a measure of concentration.

In recent years the method has been raised to what can be, in experienced hands, a high level of accuracy. Much of the credit for this is due to workers at the Eastern Regional Research Laboratory of the United States Department of Agriculture, and many of their refinements of the original method have been consolidated by the collaborative surveys organized by the Spectroscopy Committee of the American Oil Chemists' Society. A number of excellent brief reviews have been published recently, e.g. Riemenschneider (1954); Herb (1955); O'Connor (1955) and Moretti and Cheftel (1955), but it is necessary here to make a more detailed survey of the development of the method in order to illustrate the usefulness and the limitations of spectrophotometric procedures. Some of the difficulties are not peculiar to this problem and the ways of overcoming them deserve wider consideration when new applications of spectrophotometry are being devised.

Interest in the alkali isomerization of fatty acids stems from the observation

of Morton, Heilbron, and Thompson (1931), and Gillam, Heilbron, Hilditch, and Morton (1931) that the mixed fatty acids of some oils showed stronger ultra-violet absorption after saponification. The intensity of absorption increased with the time of saponification and was most marked with the more unsaturated acids (Dann and Moore, 1933). The rise in specific absorption indicated the appearance of conjugated double bonds, but iodine values showed no increase in total unsaturation; indeed, there appeared to be a fall (Dann, Moore, Booth, Golding, and Kon, 1935). Edisbury, Morton, and Lovern (1935) confirmed this, finding that the higher the temperature of saponification, the more intense was the ultra-violet absorption produced, but the greater the fall in iodine value.

The nature of these artifacts was established by Moore (1937), when he isolated from linseed oil after heating with alkali a conjugated triethenoid fatty acid of similar type to elaeostearic, with λ_{max} about 270 mμ. This pseudo-elaeostearic acid was derived from linolenic acid by migration of the double bonds to form a conjugated system. However, the isomerization was not complete; linolenic acid gave another isomeric acid with λ_{max} near 230 mμ, i.e. only partly conjugated, to give a diene chromophore. Oils containing linoleic acid showed after heating with alkali a peak near 230 mμ; here, presumably, one of the two double bonds had migrated to form a conjugated diene acid. Moore's work was confirmed by Kass and Burr (1939); their degradation studies firmly established the structure of ψ-elaeostearic acid as octadeca-10:12:14-trienoic acid.

It is usually accepted that if two double bonds are to become conjugated in this way, they must be in a 1:4-diene system (—CH=CH—CH_2—CH=CH—) and three or more bonds will conjugate only in similar extended polyallylic systems (to use the nomenclature of Hatt and Szumer, 1953). In a frequently cited paper, Farmer (1942) reported that 1:5-diene systems would not readily form conjugated structures in hot alkali, but the only substances tried were squalene, dihydromyrcene and rubber, which contain methyl branch groups in the system. These findings have been widely accepted as proof that *unsubstituted* 1:5-dienes and those with double bonds further apart will not undergo alkali-isomerization. While this is plausible on chemical grounds and may well be true, it has not been verified experimentally. There is abundant evidence that non-conjugatable dienes exist. Lemon (1944) first found that on hydrogenation linolenic acid gave rise to an octadecadienoic acid which conjugated in alkali to only a slight extent. This 'isolinoleic' acid is now known to be a mixture. The central double bond of linolenic acid has been saturated to give octadeca-9:15-dienoic acid and some shifting of the double bonds has produced a mixture containing not only the 9:15- but also the 8:14- and 10:14-isomers and perhaps others (Rebello and Daubert, 1951b). Whether any of these isomers will conjugate in alkali has not been investigated; there is the complicating factor that the 'isolinoleic acid' mixture contains some trans double bonds (Lemon and Cross, 1949; Rebello and Daubert, 1951a,b) which would at best conjugate only slowly (see p. 252).

These non-conjugatable isomers occur not only in processed material but also in natural fats. WEENINK (1956) has found that of the octadecadienoic acids in lamb caul fat, only about a third can be detected by alkaline isomerization. The non-conjugatable acids were probably formed by microbiological hydrogenation of dietary linolenic acid in the rumen.

Against the common view that only 1:4-dienes will conjugate are the claims of some Japanese workers. They have isolated from fish oils several polyunsaturated acids in which they report 1:5-systems. These acids include hiragonic (hexadeca-6:10:14-trienoic) acid (TOYAMA and TSUCHIYA, 1935a), moroctic (octadeca-4:8:12:15-tetraenoic) acid (TOYAMA and TSUCHIYA, 1935b), eicosa-4:8:12:16-tetraenoic acid (TOYAMA and TSUCHIYA, 1935c; TOYAMA and SHIMO-OKA, 1954a) and clupanodonic (docosa-4:8:12:15:19-pentaenoic) acid (TOYAMA and TSUCHIYA, 1935d; 1936). TOYAMA and SHIMO-OKA (1954b) find that all these acids will conjugate to about the same extent as the well known acids such as linolenic, arachidonic and others with double bonds interrupted by only a single methylene group. (Quantitative details are reproduced by HERB (1955).) It is clear that either 1:5-dienes do conjugate in alkali or the structures of these acids proposed by TOYAMA and co-workers are wrong.

Ability of double bonds to conjugate in alkali has been used as proof of a 1:4-diene system and as eliminating a 1:5-system in structural determinations, e.g. BALIGA and HILDITCH (1949); SILK and HAHN (1954b); MEAD and SLATON (1956). It is obviously desirable, as WHITCUTT and SUTTON (1956) have observed, that this practice be placed on a sounder basis by testing model polyenes without branch methyl groups.

On the other hand, evidence for the proposed structures of the allegedly 1:5-conjugatable acids is not easy to assess as it is mostly in the Japanese language. It should be said that other workers seem to have found only 1:4-type acids in material similar to that from which TOYAMA and collaborators have isolated only their 1:5-type. For example, SHIMO-OKA and TOYAMA (1954) found in ox liver lipid eicosa-4:8:12:16-tetraenoic acid; KLENK and DREIKE (1955) found only acids with divinylmethane structures. Other workers too have found only 1:4-type acids in fish oils (SILK and HAHN, 1954b; WHITCUTT and SUTTON, 1956; KLENK, 1954). The detailed chemistry of these polyunsaturated acids is complicated by the relative ease with which structural alterations can occur during the procedures of isolation. The comments of SILK and HAHN (1954a), SUTTON (1955), and WHITCUTT and SUTTON (1956) are well worth consideration.

There is also an anomalous report by BERGMANN and SWIFT (1951) of a hexacosadienoic acid in sponge lipids. They indicated that it was hexacosa-17:20-dienoic acid, but they failed to get conjugation of the double bonds on treatment with alkali. The structural evidence, however, is a little indirect, and BERGMANN and SWIFT say their results only 'indicate' the suggested structure. There seems a need for reinvestigation of this apparently non-conjugatable 1:4-diene acid.

Much recent work has shown that acetylenic acids undergo more complex

isomerizations in the presence of alkali than do ethylenic acids (see, for instance, JONES, WHITHAM, and WHITING (1954), and JONES, SHAW, and WHITING (1954)). The only naturally occurring acetylenic acid on which the analytical alkali-isomerization procedure has been tried and reported is ximenynic or santalbic (octadec-11-en-9-ynoic) acid. LIGTHELM *et al.* (1952) found that the original enyne system rearranged to give a conjugated triene acid, i.e. alkali had changed a —CH=CH—C≡C— grouping to —CH=CH—CH=CH—CH=CH.

A. Development of analytical method

When KASS and BURR (1939) produced ψ-elaeostearic acid from linolenic acid, they made use of the finding (EDISBURY *et al.*, 1935) that the isomerization goes much faster at higher temperatures. By using ethylene glycol as the solvent instead of ethanol, they were able to raise the working temperature. This was an important advance; alkali isomerization could now be carried out quickly enough for practical analytical work. Indeed KASS, MILLER, HENDRICKSON, and BURR (1940) (see also MILLER and BURR, 1941) used it to determine linoleic acid indirectly from the amount of conjugated isomer formed.

MITCHELL, KRAYBILL, and ZSCHEILE (1943), however, first published a detailed (and improved) method by which both linoleic and linolenic acids could be determined. Briefly, the procedure was as follows: 10 ml (11 g) of ethylene glycol containing about 6·5 per cent (w/w) potassium hydroxide were heated in a test tube in an oil bath at 180°C $\pm$ 1°C. The tubes were of standard size and were immersed in the bath to a constant depth. When the alkaline glycol had reached a temperature of 180°, a small vial, containing about 100 mg of fat or fatty acids, was dropped in. The tube was swirled three times at one-minute intervals to mix the fat with the glycol. After 25 min the tube was removed and cooled rapidly. The contents were diluted with ethanol to a suitable volume, and the extinctions determined at 234 mμ and 268 mμ.

The blank solution in the compensating cell of the spectrophotometer consisted of alkaline glycol which had been heated and diluted in exactly the same way as the solution of fat or acids.

Pure linoleic and linolenic acids were prepared by the usual bromination-debromination procedure, and their extinction values measured after isomerization under precisely the same conditions. From the standard data obtained in this way, factors could be used to calculate the linoleic and linolenic acid contents of fat samples. The extinction produced at 268 mμ by alkali treatment was considered as derived wholly from linolenic acid. The contribution of this linolenic acid at 234 mμ was then calculated; the remaining extinction at 234 mμ was assumed to be due to linoleic acid.

MITCHELL *et al.* confirmed for their conditions MOORE's (1937) findings that the formation of conjugated isomers from unconjugated acids was far from complete. Especially was this so with linolenic acid which, instead of forming conjugated triene exclusively, gave a mixture of conjugated diene and triene acids (see Fig. 7). The extinction of the conjugated triene (268 mμ) was lower than at the diene peak (234 mμ) and was less than a third of that obtainable

from complete triene conjugation: the $E_{1\text{ cm}}^{1\text{ per cent}}$ at 268 mμ of the isomerized linolenic-acid solution was under 550, the $E_{1\text{ cm}}^{1\text{ per cent}}$ of purified ψ-elaeostearic acid is around 2000. To obtain reproducible results, the temperature and the time of heating had to be kept constant. The method is thus empirical, and, despite all the later work done on it, has remained so. It suffers from many of the disabilities common to nonstoichiometric analytical procedures.

Without changing in principle the method of MITCHELL *et al.* (1943), later workers have improved and extended it in many ways. Most of the details

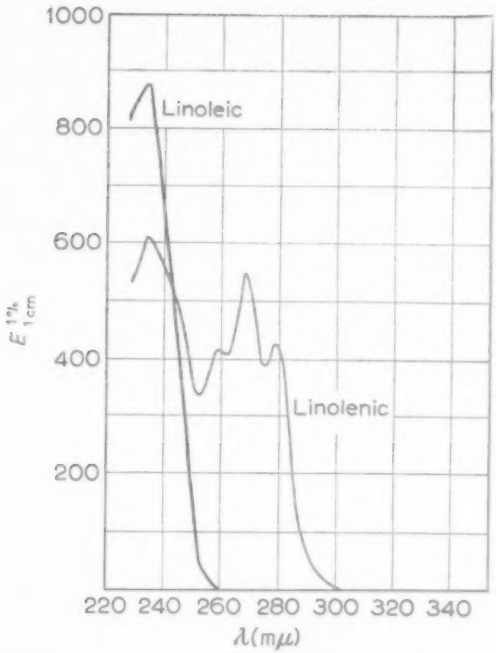

Fig. 7. Absorption spectra of linoleic and linolenic acids after isomerization in glycol containing 6·5 per cent KOH. (MITCHELL *et al.*, 1943.)

have been tested critically: the preparation of better standards of reference; variations in the time of heating; variations in the temperature; changes of solvent; concentration of alkali; and size of sample. The method has been refined and its utility broadened by the introduction of correction procedures to allow for irrelevant absorption; it has been adapted to micro quantities of material, and it has been extended to more highly unsaturated acids.

MOWRY, BRODE, and BROWN (1942) had previously studied the alkali isomerization of arachidonic acid and had shown that a conjugated tetraene system was formed. From the isomerization mixture a crystalline product was isolated which had absorption maxima at 286, 300 (main), and 315 mμ, with an inflexion at 272 mμ. BEADLE and KRAYBILL (1944) took advantage of this

to extend the method of MITCHELL *et al.* (1943) by putting a fairly pure sample of arachidonic acid through the same procedure, and recording the factors obtained. Since then attempts have been made to use the method for estimating acids with five and six double bonds, but they have not reached the level of accuracy attainable with di- and tri-enoic acids.

In order to survey the amendments made to the basic procedure of MITCHELL *et al.* (1943), it is convenient to consider separately the features in which variations and improvements have been suggested, namely: the time of heating, the temperature, the solvent used for isomerization, standard preparations of acids, correction procedures for irrelevant absorption, and the concentration of alkali used.

B. Time of heating

When most (unconjugated) unsaturated fatty acids are heated in alkali, two distinct processes occur. Conjugated polyene acids are formed by rearrangement of previously insulated double bonds, and, at the same time, the newly formed conjugated systems tend to disappear as a result of other reactions which are not fully understood. Acids containing conjugated double bonds are not very stable at best, and certainly are not when treated with very hot alkali. Their instability is related to the length of the conjugated system. When subjected to the treatment used for alkali isomerization of unconjugated acids, conjugated dienoic acids are relatively stable, conjugated trienoic acids are partly decomposed (HILDITCH, MORTON, and RILEY, 1945; BRICE and SWAIN, 1945—see Fig. 8), and conjugated tetraenoic acids largely decomposed (RILEY, 1950).

According to MARUYAMA and SUZUKI (1931) not all conjugated dienoic acids are equally stable in such conditions. They reported that octadeca-8:10-dienoic acid on heating in ethanolic potassium hydroxide gave stearolic (octadec-9-ynoic) acid almost quantitatively. It seems possible that this claim may be in error. In the same paper, sorbic acid was reported to rearrange to hex-3-ynoic acid, by a presumably similar reaction, but JONES, WHITHAM, and WHITING (1954) have been unable to repeat this with sorbic acid.

Some uncommon conjugated acids behave unusually when subjected to alkali isomerization. Thus deca-2:4-dienoic acid, with its two ethylenic links conjugated to the carboxyl group, produces a complex mixture of conjugated dienoic acids (CROSSLEY and HILDITCH, 1952). A preliminary report by KAJANNE (1950) of apparently similar behaviour by a fatty acid with four double bonds found in pine wood has since been withdrawn (KAJANNE, 1953).

These uncommon fatty acids are, however, exceptional. The destruction of most of the more complex, conjugated polyenes in alkali is not a deconjugation by mere reversal of alkali isomerization. HOLMAN and BURR (1948) found that the triene peak of ψ-elaeostearic acid became weaker on heating in potassium hydroxide-glycol without any compensatory increase of diene conjugation. HERB and RIEMENSCHNEIDER, 1952, and HAMMOND and LUNDBERG, 1953, have found more highly unsaturated acids to behave similarly—see Fig. 16.) Poly-

merization and cyclization probably account mainly for the loss of triene conjugation. BRADLEY and RICHARDSON (1942) subjected tung oil acids—mainly the naturally conjugated elaeostearic—to an alkaline isomerization procedure and distilled the acids at the end; the alkali treatment had caused a big increase in the nonvolatile residue.

From the time it is produced, the conjugated isomer is thus exposed to destructive processes, so that the concentration of conjugated product derived from any one acid will reach a maximum after a certain time and will then fall away

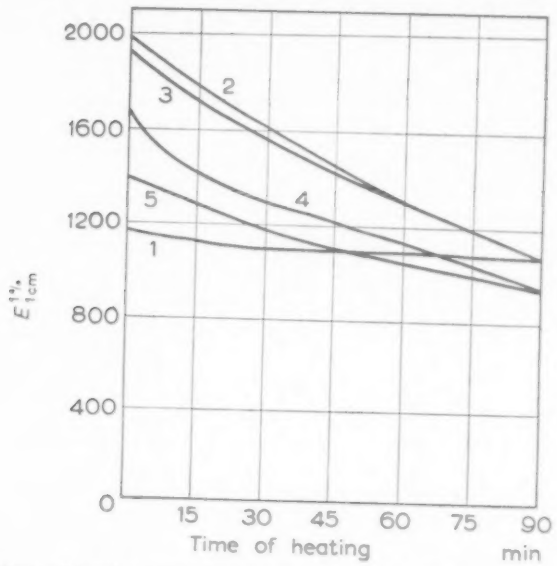

Fig. 8. Effect of heating conjugated unsaturated acids in alkaline glycerol. 1, 10:12-linoleic acid; 2, β-elaeostearic acid; 3, ψ-elaeostearic acid; 4, α-elaeostearic acid; 5, tung oil (BRICE and SWAIN, 1945).

(see Fig. 9). For analytical purposes, the method will be most sensitive if the extinction is read at the time when it is at its greatest. If, however, the extinction at that period is changing rapidly with time, it may be better to take a reading when the extinction is lower, but more steady, i.e. on a flatter part of the curve obtained by plotting extinction against time (see Fig. 9). Should a sample then for any reason deviate slightly from the normal in respect of speed of isomerization, the error introduced will be smaller; sensitivity may have to be sacrificed for accuracy. Further, the shape of the isomerization curve (extinction plotted against time) is changed by factors such as the temperature of heating, the concentration of alkali and the size of the fat sample. All these will influence the selection of a time for taking a reading.

In spite of these variables, it is not difficult to pick a suitable isomerization time when single unsaturated acids are being assayed under fixed conditions. With mixtures of acids the problem is considerably more complicated. Each acid has its own isomerization curve (see Fig. 9) and each is affected differently

by variations in the experimental conditions. Spectrophotometric readings made at a selected time may give trustworthy results for one component of a mixture, but erroneous ones for others. Two alternative courses can be pursued: either observations can be made at more than one time, or a single time of isomerization may be decided upon by compromise.

For the determination of mixtures of linoleic and linolenic acids—the typical problem to which alkali isomerization has been applied—Hilditch *et al.* (1945)

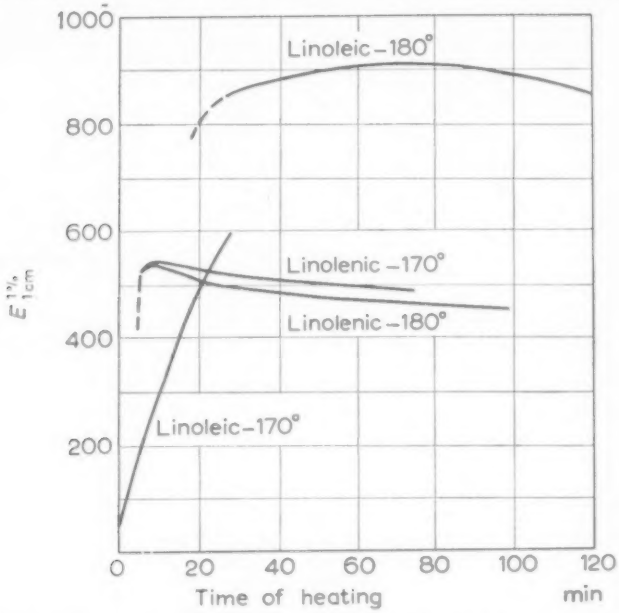

Fig. 9. Extinction developed by linoleic and linolenic acids at 234 mμ and 268 mμ respectively when heated for varying times at 170° and 180° in 6·5 per cent KOH/glycol (Hilditch *et al.*, 1945).

suggested taking spectrophotometric readings at two times. The method has been used regularly and developed further (Hilditch, Patel, and Riley, 1951). It can easily be combined with the use of different reaction temperatures (see later). Its disadvantage is that extinction readings for the linolenic isomers are taken at a time when the whole absorption spectrum is changing quickly enough to make errors more likely.

Most workers have preferred to take only one reading, usually after 25–45 minutes, e.g. Mitchell *et al.* (1943); Spectroscopy Committee, American Oil Chemists' Society (1949, 1951, 1953); Brice, Swain, Herb, Nichols, and Riemenschneider (1952). Brice *et al.* (1952) regard 45 minutes as optimal for the predominating all-cis acids, although the Spectroscopy Committee of the American Oil Chemists' Society (1953) found this no better than 25 minutes. When other isomers are present, different times may have to be used (Jackson, Paschke, Tolberg, Boyd, and Wheeler, 1952).

In isomerizations using stronger alkali (see later), shorter heating times are necessary, especially for the highly unsaturated acids; HAMMOND and LUNDBERG (1953) found that hexaenoic acids attained maximum conjugation in about 4 minutes (see Figs. 15 and 16). If a single heating time is chosen, it can only be a compromise; it should be the one which will give the best results under the stated conditions, and for the sample assayed. Although by taking readings after a single time interval successful assays of vegetable oils containing all-cis di- and tri-enoic acids can be achieved, it may be necessary to choose two or more time intervals when mixtures are being assayed which contain unnatural isomers or highly unsaturated acids.

C. Temperature

Choice of the temperature of isomerization raises essentially the same issues; the higher the temperature, the quicker the development of conjugation, and the faster the destruction of the newly formed isomers. But the various processes are not affected to the same extent and so for any one acid there will be an optimal temperature at which the yield of selectively absorbing isomer will be at its highest. Again, this temperature unfortunately differs among acids. HILDITCH *et al.* (1945), for instance, found that linoleic acid isomerized only slowly at 170°, and even at 180° took about 60 minutes to reach its maximum. By contrast, linolenic acid under the same conditions attained its peak within 15 minutes at 170° (see Fig. 9). At temperatures both above and below this, less intense ultra-violet absorption indicated a smaller yield of conjugated isomer.

To get more accurate results with linoleic and linolenic acids, HILDITCH *et al.* (1945) proposed carrying out two similar determinations at different temperatures: at 180° for linoleic and at 170° for linolenic acid. In principle, this has much to commend it; we have already mentioned that isomerization of linolenic acid is far from complete under the usual conditions, and it would seem desirable to counteract this by securing the maximum attainable conjugation. The procedure of HILDITCH *et al.* (1945), as later modified by HILDITCH *et al.* (1951), does give higher extinction readings than do other procedures based on the use of weak alkali. However, most workers have preferred the simpler procedure of isomerizing at one selected temperature, which indeed gives very good, and possibly more reproducible, results.

D. Solvent for isomerization

MITCHELL *et al.* (1943) used ethylene glycol as the solvent in the isomerization mixture and obtained satisfactory results with mixtures containing substantial proportions of linoleic acids. With fats low in polyunsaturated acids, BRICE and SWAIN (1945) got inaccurate figures; on heating in air, the potash-glycol mixture developed strong ultra-violet absorption, variable, unfortunately, even between the test and blank samples run side by side. This kind of error was greatly reduced by substituting glycerol for glycol; glycerol could be heated in air without developing strong absorption.

O'Connor, Heinzelman, Caravella, and Bauer (1945) overcame the difficulty in another way by protecting the potash-glycol mixture with nitrogen. This improved the transparency of the blank solution even more than did the glycerol (in air) modification. It has been found that, for high accuracy, it is necessary to protect the KOH-glycol mixture not only during heating but also during storage before use.

Glycol has remained in general favour. Some workers have found glycerol unsatisfactory for their purposes, e.g. McDowell (1953) and Hilditch *et al.* (1951) reported that they got less concordant results using glycerol than with glycol (even without nitrogen protection) when substantial amounts of linoleic and linolenic acids were present. Other workers, however, have continued to use glycerol and appear to get slightly better results than with glycol (Brice *et al.*, 1952). Collins and Sedgwick (1956) have also used glycerol in their rapid method for soya bean oil. Both glycol and glycerol are accepted in standard procedures; there is perhaps not much to choose between the two solvents.

Although glycol and glycerol are the most widely used solvents, others can be used: water (Bradley and Richardson, 1942; Berk, Kretchmer, Holman, and Burr, 1950); diethylene glycol (von Mikusch, 1949); and Cawley (1944) has reported that glycol ethers are very effective.

E. Calibration by means of experiments on purified acids

In order to ascertain how linoleic and linolenic acids behave under standardized conditions of alkali isomerization, Mitchell *et al.* (1943) prepared pure esters from natural oils. This procedure involved separating the acids through their polybromide derivatives which were then debrominated. Esters prepared by bromination and debromination were used as standards by all the early workers (including Beadle and Kraybill, 1944, when they extended the method to arachidonic acid) as being the best available at the time.

Brice, Swain, Shaeffer, and Ault (1945) first applied the procedure to a wide range of fats and the results obtained appeared to be slightly wrong. Bromination and debromination of acids is known to produce a mixture of geometrical isomers and Brice *et al.* pointed out that these isomers might not all conjugate at the same rate. Nichols, Riemenschneider, and Herb (1950) have shown this view to be correct. 9-cis-12-cis-Linoleic acid, which predominates in nature, isomerizes much faster than its trans-trans isomer, linolelaidic acid. Jackson *et al.* (1952) have investigated these differences in more detail (Fig. 10).

Nichols, Herb, and Riemenschneider (1951) considered alkali isomerization as a prototropic shift mechanism and deduced, on theoretical grounds, which isomers of conjugated linoleic acid should be produced. They concluded that either the 9–10 or the 12–13 double bond of linoleic acid may migrate to give a mixture of 9:11- and 10:12- conjugated linoleic acids. The double bond which does not shift retains its configuration; that which does move may change. When the bond involved in the shift is cis, the new bond formed is trans. When

the bond involved in the shift is trans, the bond formed can be either cis or trans. 'Natural' 9-cis:12-cis-linoleic acid will therefore give a mixture of 9-cis:11-trans-linoleate and 10-trans:12-cis-linoleate. Unnatural cis-trans isomers will give different mixtures. The experimental findings of NICHOLS *et al.* (1951) and of JACKSON *et al.* (1952) indicate that these predictions are probably true. As geometrical isomers of conjugated linoleic acids will have different extinction coefficients, not only will cis-trans isomers of 9:12-linoleate conjugate at different rates but also the final equilibria will not be the same (see Fig. 10).

However, during the periods of time used for most isomerizations the predominant differences between isomers will be due to their speeds of conjugation. Debrominated linoleic acid, which is a mixture of isomers, will therefore not

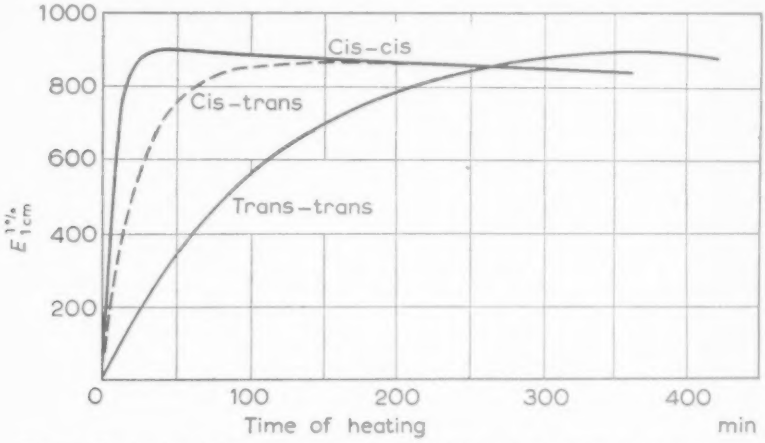

Fig. 10. Extinction developed by isomers of linoleic acid (cis-cis at 233 mμ, others at 231 mμ) on heating in 6·5 per cent KOH/glycol at 180° (JACKSON *et al.* 1952).

isomerize at the same speed as the naturally occurring cis-cis-acid of vegetable oils. This can be shown experimentally, not only for linoleic, but also for linolenic and arachidonic acids (BRICE *et al.*, 1952). By the use of physical rather than chemical methods of separation pure samples of linoleic and linolenic acids (RIEMENSCHNEIDER, HERB, and NICHOLS, 1949) and later, arachidonic acid (HERB, RIEMENSCHNEIDER, and DONALDSON, 1951) have been isolated in their all-cis forms. These acids have been used to establish new calibrations much more serviceable than those obtained earlier with debromination acids. Discrepancies previously found between spectroscopic analyses and iodine values were often greatly reduced (BRICE *et al.*, 1952). HILDITCH *et al.* (1951) have similarly amended the method of HILDITCH *et al.* (1945).

The use of standard data obtained with natural acids has improved the accuracy of the method as applied to unprocessed vegetable oils where the fatty acids retain their all-cis configuration. There is, however, an increasing amount of evidence that animal fats, notably those of ruminants, contain a proportion of trans isomers, and possibly more important, that processing of oils may cause

isomerization of the fatty acids. The application of standard methods to such mixtures can give unexpected results.

To take an extreme but uncomplicated example: JACKSON *et al.* (1952) found that after thermal polymerization of methyl linoleate, unpolymerized linoleate can be recovered, which does not assay as such by the ordinary spectroscopic procedures; the cis-cis-linoleate has been isomerized. Similarly, the non-conjugated octadecadienoic acid obtained by dehydration of ricinoleic acid (from castor oil) contains a mixture of cis-trans isomers. To get accurate results on such mixtures containing uncommon isomers, the alkali treatment must be carried on for a longer time to allow optimal conjugation of the slower reacting components: 285 minutes is suggested by JACKSON *et al.* (1952). The variations in rates of conjugation can be turned to good account; from the speed of conjugation of a mixture, the approximate proportions of each isomer can be estimated (JACKSON *et al.*, 1952). Unfortunately this cannot apply when linolenic acid is present owing to the destruction of conjugated triene during the long isomerization period (CORNWELL, BACKDERF, WILSON, and BROWN, 1953). A correction procedure to apply to oils in which these less common isomers are present together with linolenic acid would be cumbersome and inaccurate.

NICHOLS *et al.* (1951) predicted the isomers to be expected from alkali isomerization of cis-cis linoleate. These predictions are true to the extent that a mixture of cis-trans isomers is produced, but this approach is probably less reliable for the more unsaturated acids. The rules of NICHOLS *et al.* (1951) indicate that when natural linolenate is isomerized to ψ-elaeostearic (octadeca-10:12:14-trienoic) acid, the 10-trans:12-cis:14-trans-isomer should be formed. PASCHKE, TOLBERG, and WHEELER (1953) investigated the infra-red absorption of ψ-elaeostearic acid and identified the structure as all-trans. It may be that the predicted mono-cis isomer is formed first and changes to the all-trans isomer under the influence of heat and alkali. KASS (1944) has reported that a mixture of isomers is produced, and pure α- and β-elaeostearic acids are known to isomerize during alkali treatment (O'CONNOR *et al.*, 1953). Certainly if a mixture were formed, the all-trans, which is the least soluble isomer, would be the one to be isolated. Although the rules of NICHOLS *et al.* (1951) need not be incorrect, they may fail to predict the composition of a final mixture because they do not take into account secondary isomerizations.

F. Correction procedures

The pioneer method of MITCHELL *et al.* (1943) was designed for the estimation of linoleic and linolenic acids in oils containing substantial amounts of these components. The conjugated products were determined from the gross extinctions at 234 mμ and 268 mμ. The extension to arachidonic acid (BEADLE and KRAYBILL, 1944) was essentially similar, i.e. it was assumed that the extinctions in the region 230–320 mμ before alkali isomerization were negligible when compared with those shown at the peaks developed by alkali treatment. This assumption is valid for oils rich in polyunsaturated acids, but where only small proportions are present the original method is unserviceable.

Brice and Swain (1945) first introduced correction procedures, to allow the method to be applied to fats with only small amounts of polyunsaturated acids. This was a much-needed development; it is for just those fats that the older methods (e.g. using thiocyanogen) are least satisfactory. Alkali isomerization of such fats induces a relatively small increase in extinction; a substantial proportion of that present after alkali treatment will have been there in the original fat. Small amounts of pre-existing absorption, which can be ignored in linoleic- and linolenic-rich oils, must therefore be taken into account. In some fats, an appreciable proportion of conjugated diene material is present before isomerization; both before and after isomerization some of the observed extinction near 230 mμ will be contributed by COOR groups; and an appreciable proportion of the observed extinction near 268 mμ and 315 mμ may be due to irrelevant background absorption.

All three contributions are allowed for in the procedure of Brice and Swain (1945), which, although lengthy, is in principle fairly simple. Before isomerization the fat is examined spectroscopically for conjugated di-, tri-, and tetraenoic acids. The extinction at 232 mμ is assumed to be fully accounted for by that of conjugated diene acids plus the contribution of carboxyl groups. The latter will be the same as that found with specimens of pure stearic acid or methyl stearate. When this has been subtracted, the remaining extinction at 232 mμ is taken to represent that of conjugated dienoic acid, and the amount present is calculated from the extinction values of pure conjugated dienoic acids.

The conjugated trienoic acids are determined from the extinction at 268 mμ. This is composed of the true absorption of conjugated trienoic acid plus irrelevant background absorption. To allow for the latter, Brice and Swain (1945) devised a procedure which has since been used widely. Its principle is illustrated by Fig. 11. β-Elaeostearic acid is taken as a typical conjugated trienoic acid. Its extinction is measured at the peak (d) and at the neighbouring minimum on the short wavelength side (e)—in this case a difference of 6 mμ. The extinction is then read at (f), a similar wavelength interval above λ_{max}. For β-elaeostearic acid in Fig. 11, 262, 268, and 274 mμ were the wavelengths chosen. The average of the extinctions of (e) and (f) will give the point (g). If k_x represents the specific extinction coefficient at x mμ, $d - g = k_{268} - \frac{1}{2}(k_{262} + k_{274})$, which is known as Δk. The extinction at λ_{max} (k_{268}) divided by Δk gives the value 2·8, i.e. $k_{268} = 2{\cdot}8\ [k_{268} - \frac{1}{2}(k_{262} + k_{274})]$. That is, the extinction of β-elaeostearic acid may be determined indirectly from Δk. The assumption then made by Brice and Swain (1945) is that over this narrow range under investigation (here, 262–274 mμ) any irrelevant absorption is linear. If so, although such irrelevant absorption will increase the gross extinction at 268 mμ, it will not change Δk. Determination of Δk will therefore enable the true β-elaeostearic acid content to be calculated.

Brice and Swain (1945) were thus able to estimate in the presence of considerable background absorption small quantities of pre-existing conjugated trienoic and, by means of a similar procedure, tetraenoic acids. They assumed these acids to have absorption spectra identical with those of the β-elaeostearic

acid of BRICE and SWAIN (1945) and the isomerized arachidonic acid prepared by BEADLE and KRAYBILL (1944) and MOWRY *et al.* (1942). These assumptions are not fully valid but in fats containing only small amounts of conjugated tri- and tetra-enoic acids, the errors introduced will not be serious.

In the procedure of BRICE and SWAIN (1945), the extinction was again measured at selected wavelengths after alkali isomerization. The differences between the pre-existing conjugation and that found after the alkali treatment

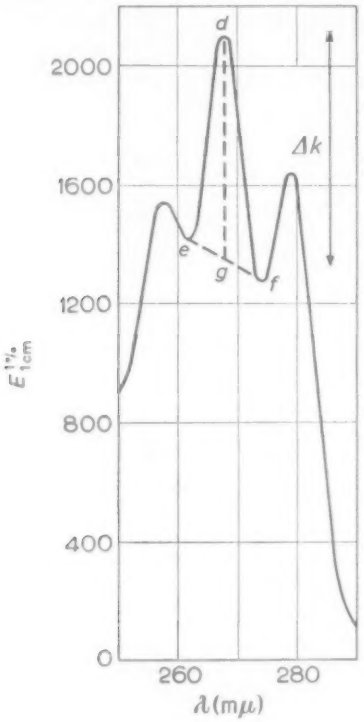

Fig. 11. Correction for irrelevant absorption: β-elaeostearic acid (BRICE and SWAIN, 1945)—see text.

are assumed to be due to the newly formed conjugated acids. Corrections are made at 232 mμ for the differing absorptions of the ester links in the fat and the carboxyl groups of the soaps formed from them. In determinations of tri- and tetra-enoic acids, irrelevant absorption is allowed for by a procedure similar to that used for preconjugated acids, except that the Δk value for alkali-isomerized trienoic acid is different from that for β-elaeostearic acid. The extinction figures thus obtained were corrected for overlapping absorption.

These are the principles of the method of BRICE and SWAIN (1945)—numerical details are given in their paper—and all subsequent correction procedures have been based on theirs. Various refinements and modifications have been suggested, partly to simplify the fairly lengthy calculations necessary and partly to improve

accuracy (Spectroscopy Committee, American Oil Chemists' Society, 1949, 1951, 1953, 1955; Brice *et al.*, 1952; O'Connor, Stansbury, Damaré, and Stark, 1952; May, 1952).

Use of correction procedures has increased the scope and, in many instances, the accuracy of methods employing alkali isomerization, but a survey of the amendments subsequently necessary shows that correction procedures must be used with caution. Later work has, indeed, tended towards the elimination of many steps of the original procedure of Brice and Swain. There is abundant evidence that application of the full correction procedure to some fats, far from increasing accuracy, can worsen it. This is particularly noticeable in fats containing substantial proportions of unconjugated tri- and tetra-enoic acids. To measure Δk values of these acids, it is, of course, necessary to determine the extinction at a sharp maximum and at one, or two, sharp minima. In practice it is not easy to get correct readings; big deviations are produced by differences in slit width and by small instrumental inaccuracies in wavelength. Brice *et al.* (1952) show how, in oils containing large quantities of, say, linolenic acid, these factors can introduce far larger errors than would persist if no correction for irrelevant absorption were applied.

It is often advisable, then, not to correct observed extinctions. Brice *et al.* (1952) and O'Connor *et al.* (1952) have advocated the omission, for many oils, of steps such as (1) the determination of preconjugated acids and (2) the use of the correction procedure for tri- and tetraenoic acids when more than small amounts are present ($E^{1\text{ per cent}}_{1\text{ cm}} > 10$). Some of the suggestions of Brice *et al.* (1952) have been incorporated into the approved method of the American Oil Chemists' Society (Spectroscopy Committee, 1953).

To sum up: in a method as empirical as is alkali isomerization, the use of a correction procedure fails with many plant oils to result in any advantage. Even when accuracy is improved, the gain frequently does not justify the extra labour necessary. The procedure of Brice and Swain (1945) was introduced to deal with fats containing small quantities of polyunsaturated acids, for which it has proved of great use. Its application, however, to oils rich in these acids is unduly cumbersome and can lead to erroneous results.

The Brice and Swain (1945) correction procedure allowed for the presence of small amounts of conjugated acids in the original oil. Where large amounts exist, as in some of the drying oils, other difficulties arise. Fig. 8 shows that although diene acids are fairly stable in the conditions of alkali isomerization, trienoic acids of the elaeostearic type are not. When these conjugated acids are present initially, it is necessary to make allowance for the partial loss of their selective absorption during the heating process. This allowance is not simple to evaluate. The idea was tried out by Hilditch *et al.* (1945) on tung oil (which contains a high proportion of elaeostearic acid), but later work (Hilditch and Riley, 1946) showed that some of the results obtained were grossly wrong. Hilditch and Riley (1946) got far better results by first separating the mixed fatty acids into several groups by crystallization, thus removing the elaeostearic acid.

Preliminary fractionation is useful in the analysis of many fat samples which contain interfering substances. For example, PRIVETT and LUNDBERG (1951) similarly overcame the difficulties of analysing fats which had undergone some autoxidation. Methods involving separation by crystallization, followed by analysis of each fraction, are however too lengthy for routine determinations of unconjugated unsaturated acid in oils which also contain conjugated acids. O'CONNOR *et al.* (1953) have been able to develop a much simpler method for the determination of linoleic acid in tung oil. It is based on the same principle as the method of HILDITCH *et al.* (1945), but makes use of the finding of HILDITCH and RILEY (1946) that elaeostearic and linolenic acids seem not to occur naturally in the same oil. It also uses better constants. O'CONNOR *et al.* (1953) found that α- and β-elaeostearic acids (most commercial tung oils contain varying proportions of the α and β acids) behave differently when put through the alkali isomerization process. Their method involves determination of the proportions of α and β acids present before and after isomerization and correction of the extinction at 233 mμ for the presence of elaeostearic acids and of polymeric material formed during the alkali treatment. This method has been used by PLANCK, PACK, HEINZELMAN, STANSBURY, and O'CONNOR (1953) for determining the fatty acid composition of partially hydrogenated tung oils. Nevertheless, high accuracy is not to be expected from methods of this type with tung oil (see the results of a collaborative survey by the Spectroscopy Committee of the American Oil Chemists' Society, 1955). As mentioned previously (p. 234) the initial determination of the proportions of α- and β-acids is not easy. There is also a report (Spectroscopy Committee of the American Oil Chemists' Society, 1956) that the constants at present in use for this method need amendment.

Kamala oil, a possible substitute for tung oil, also presents difficulties as it too contains a conjugated trienoic acid: kamlolenic. GUPTA, GUPTA, and AGGARWAL (1954) have used for kamala oil a modification of the method of HILDITCH *et al.* (1945). It is probably somewhat inaccurate, but substantial errors seem inevitable owing to the rapid gelling of the oil, which is no doubt a reflection of its unusual constitution (O'NEILL, DENNISON, and AHLERS, 1954).

It should be mentioned that, although HILDITCH and RILEY (1946) found that elaeostearic and linolenic acids do not usually occur together, small amounts of elaeostearic acid have been found in commercial samples of stillingia oil, which does contain linolenic acid (CROSSLEY and HILDITCH, 1950). Stillingia oil is in any case unusual in that it contains deca-2:4-dienoic acid which behaves oddly on alkali isomerization (see p. 248). To analyse the oil, CROSSLEY and HILDITCH (1950) had first to separate it into different fractions, removing the interfering acids.

G. Concentration of alkali

Although other substances could be used—sodium, caesium and lithium hydroxides or alkoxides (CAWLEY, 1944; DAVENPORT, BIRCH, and RYAN, 1956)—potassium hydroxide has been almost exclusively employed as the isomerizing agent. Most workers have used concentrations between 6 per cent

and 11 per cent; under these conditions (which we shall call *weak-alkali* in contrast with the *strong-alkali* procedure described later), the formation of conjugated isomers from unconjugated acids is incomplete. Recent work has shown that a standard weak-alkali treatment produces from linoleic acid about 95 per cent of the theoretically possible diene conjugation (NICHOLS *et al.*, 1951); but, as mentioned already, the yield of ψ-elaeostearic acid from linolenic acid is poor. Yields of tetra- and penta-conjugated polyene chromophores from

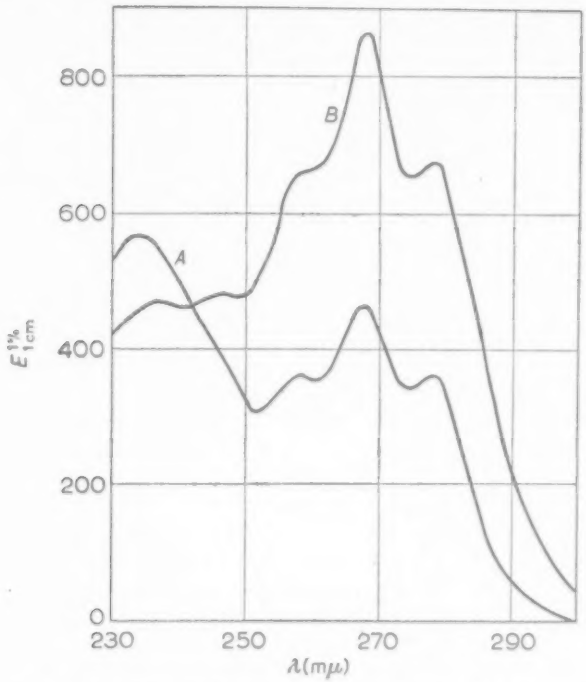

Fig. 12. Absorption spectra of linoleate after isomerization by (*A*) a weak-alkali procedure and (*B*) 21 per cent KOH in glycol (HERB and RIEMENSCHNEIDER, 1952).

the more highly unsaturated acids are even smaller. The longer conjugated polyene chromophores have very high extinction coefficients which would allow accurate determinations of very small amounts of unconjugated acids, if only they could be more fully isomerized. As such acids are usually present in fats only in small amounts, more sensitive methods for assaying them are very desirable.

The presence of polyunsaturated acids cannot safely be ignored; calculations on fats containing penta- and hexa-ene acids, using only factors for di-, tri-, and tetra-ene acids, frequently give negative values for di- and tri-enoic acids (see, for instance, RIECKEHOFF, HOLMAN, and BURR, 1949; O'CONNELL, LIPSCOMB, and DAUBERT, 1952; HERB and RIEMENSCHNEIDER, 1953; Spectroscopy Committee, American Oil Chemists' Society, 1953). Even when positive values

are obtained, they must be considered unreliable (see the example given by HERB, 1955). All variations of temperature and of the time of heating have failed to raise the efficiency of isomerization of polyunsaturated acids. The only effective method yet found is an increase in alkali concentration. HOLMAN and BURR (1948) found that concentrations of alkali higher than the 6–11 per cent commonly used had a negligible effect on the maximum diene conjugation induced with linoleic acid (which, of course, could not be increased greatly) but much more intense absorption was produced from linolenic and arachidonic

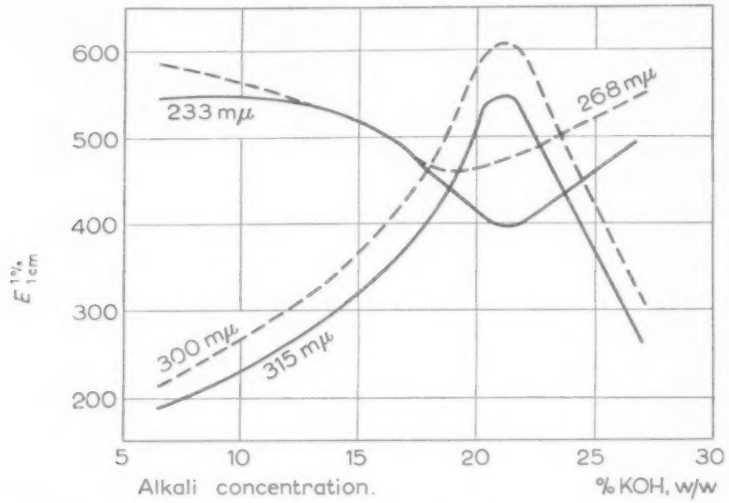

Fig. 13. Extinctions produced by isomerization of arachidonate in glycol containing varying amounts of potash: heated at 180° for 15 min (HERB and RIEMENSCHNEIDER, 1952).

acids (see Fig. 12). The induced extinction increased with alkali concentration to an optimum, beyond which the yield decreased. The best concentration of potash to isomerize tri- and tetra-ene acids is just over 20 per cent. HERB and RIEMENSCHNEIDER (1952) later established optimal conditions for producing tetraene conjugation from arachidonic acid. In their procedure (which differs slightly from that of HOLMAN and BURR, 1948) the alkali concentration for maximum yield is quite critical; even small deviations from the optimum cause big falls in the resulting extinction (see Fig. 13).

HOLMAN and BURR (1948) did not carry out a rigorous investigation on the more highly unsaturated acids owing to lack of suitable standards, but the method was later extended to penta- and hexa-ene acids after the isolation of methyl esters of eicosapentaenoic, docosapentaenoic (HERB, WITNAUER, and RIEMENSCHNEIDER, 1951; HERB and RIEMENSCHNEIDER, 1952) and docosahexaenoic acids (HAMMOND and LUNDBERG, 1953; ABU-NASR and HOLMAN, 1954). With these also, high concentrations of alkali are very effective in producing penta- and hexa-ene conjugation (Figs. 14 and 15).

This work of HOLMAN and BURR (1948) made further advance easier. The

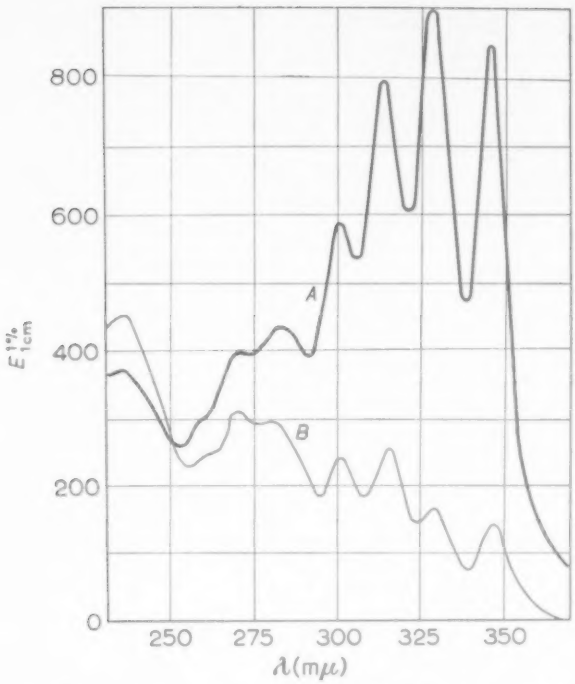

Fig. 14. Absorption spectra of eicosapentaenoate after isomerization by (*A*) 21 per cent KOH in glycol and (*B*) a weak-alkali procedure (HERB and RIEMENSCHNEIDER, 1952).

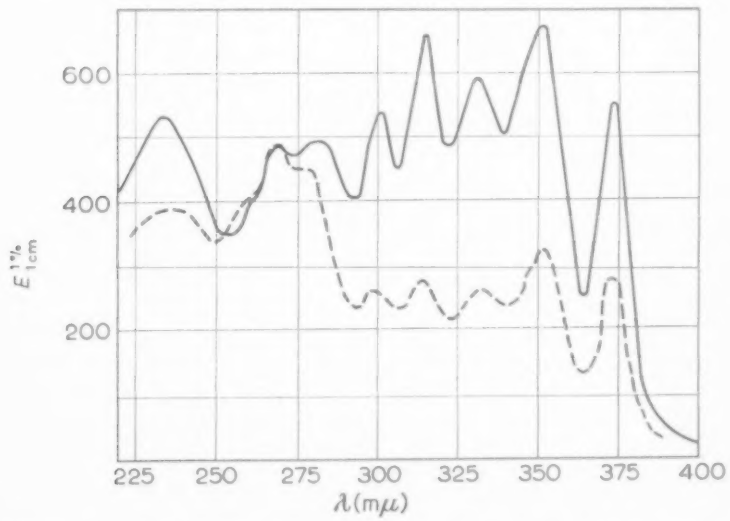

Fig. 15. Absorption spectra of docosahexaenoate after isomerization in 21 per cent KOH/glycol for 4 min (solid line) and 15 min (broken line) (HAMMOND and LUNDBERG, 1953).

improved isomerization achieved by the use of more concentrated alkali opened up an extension of the method to the more unsaturated acids. It could also be used on a micro scale which is essential for many biochemical investigations. Nevertheless the greatly increased extinctions found by HOLMAN and BURR (1948) and by later workers are still very low relative to those of the naturally conjugated acids, e.g. $E^{1\text{ per cent}}_{1\text{ cm}}$ (300 mμ) for the isomerized arachidonate solution is 622 whereas $E^{1\text{ per cent}}_{1\text{ cm}}$ for the parinaric acids at this wavelength is about 3000.

If only 1:4-dienes conjugate readily in alkali and 1:5-dienes do not, this low yield seems inevitable. RIEMENSCHNEIDER (1954) gives linolenic acid as an example; assuming a prototropic shift mechanism, he shows that in the first stage of isomerization of linolenic acid (I), four isomers (II–V) will be formed with a conjugated diene group.

$$CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH \quad \text{(I)}$$

$$CH_3CH_2CH_2CH{=}CHCH{=}CHCH_2CH{=}CH_2(CH_2)_7COOH \quad \text{(II)}$$

$$CH_3CH_2CH{=}CHCH{=}CHCH_2CH_2CH{=}CH(CH_2)_7COOH \quad \text{(III)}$$

$$CH_3CH_2CH{=}CHCH_2CH_2CH{=}CHCH{=}CH(CH_2)_7COOH \quad \text{(IV)}$$

$$CH_3CH_2CH{=}CHCH_2CH{=}CHCH{=}CHCH_2(CH_2)_7COOH \quad \text{(V)}$$

Of these, two (II and V) can form a conjugated triene, but the other two (III and IV) contain a 1:5-diene structure and will not readily isomerize further. Consequently a mixture of triene and diene chromophores will be formed.

If this is a correct interpretation of the reaction mechanism, the yield of polyunsaturated chromophores from acids more unsaturated than linolenic will obviously become much smaller; the fact that the 'yields' from such acids are always poor lends support to the assumption that 1:5-dienes do not conjugate.

HERB *et al.* (1951) and HERB and RIEMENSCHNEIDER (1952) were first to prepare pure specimens of pentaene acids and were able to extend the strong-alkali method. The results were both rewarding and disappointing. The concentrated alkali was far more effective in forming the pentaene chromophore with methyl eicosapentaenoate (see Fig. 14); but the extinction developed by methyl docosapentaenoate was much less than that obtained from the C_{20} acid. To get accurate results on any fat sample, it is therefore necessary to know the chain lengths of any pentaene acids present. HERB and RIEMENSCHNEIDER (1952) advise a working assumption of equal quantities of C_{20} and C_{22} pentaene acids since HERB *et al.* (1951) found it to apply to cattle suprarenal lipid. That equal proportions occur in all fats may however be a rash assumption.

It is now apparent that variations with chain length are not confined to the pentaenes, but are also shown by other acids. SILK and HAHN (1954b) found hexadeca-6:9:12:15-tetraenoic acid (from pilchard oil) when isomerized with strong alkali gave a peak at 315 mμ nearly 50 per cent higher than did the C_{20} arachidonic acid reported by HERB and RIEMENSCHNEIDER (1952). Moreover,

arachidonic acid gave not only tetraene, but also tri- and diene peaks, whereas hexadecatetraenoic acid gave only those of the conjugated tetraene. In the analysis of mixtures containing tetraene acids, diene and triene absorption produced by only partial conjugation is always corrected for. If, however, hexadecatetraenoic acid rather than arachidonic is present, such a correction will patently lead to errors.

The behaviour of the octadecatetraenoic acid found in sardine oil differs from that of these C_{16} and C_{20} tetraene acids. In weak-alkali it gives a much higher peak near 315 mμ than does arachidonic, but unlike hexadecatetraenoic acid, it shows diene and triene peaks indicative of only partial conjugation (Toyama and Shimo-oka, 1954b). However, this specimen was prepared by bromination and debromination and probably contained some isomers.

As hexadeca-6:9:12:15-tetraenoic acid shows no di- and tri-ene, but only tetraene conjugation, the mechanism of isomerization proposed by Riemenschneider (1954) cannot be operating for it. Sutton and Stubbs (private communication) suggest that the unusual vinyl end group is exercising a directing effect on the migration of the double bonds.

These disconcerting variations in behaviour with chain length exhibited in the penta- and tetra-ene acids raise the question whether similar discrepancies arise between the C_{18} linoleic and linolenic acids and the unusual di- and trienoic acids of other chain lengths between C_{16} and C_{22}. There have been many reports of such acids from a number of sources, but usually only of small quantities so that even if variations do occur with chain length, no substantial errors are introduced in the analysis of the total fats. However, many workers determine the di- and tri-ene content of fractions relatively rich in these acids by alkaline isomerization, using factors based on linoleic and linolenic acids, e.g. Baliga and Hilditch (1949); Heyes and Shorland (1951); Hatt and Szumer (1953). There is no certainty that this practice is fully justifiable. It is probable that it is in many cases, for Paschke and Wheeler (1954) determined the uncommon C_{16} di-, tri- and tetra-enoic acids and a C_{18} tetraenoic acid from the fat of the alga *Chlorella* which contains remarkably large amounts of these acids. Their total acid figures, obtained by adopting the standard conversion factors for linoleic, linolenic and arachidonic acids, agreed very well, even surprisingly well, with the iodine value. It seems clear that weak-alkali isomerization is less likely than strong-alkali to bring out differences between acids of equal unsaturation but different chain lengths. In the trienoic acids at least, the slower rates of conjugation and destruction of the chromophore certainly tend to lessen differences. Hexa-7:10:13-trienoic (Heyes and Shorland, 1951) and hiragonic acid (Toyama and Shimo-oka, 1954b) seem to behave much the same as linolenic acid in weak alkali. In strong alkali, differences are apparent between linolenic acid and hiragonic (Toyama and Shimo-oka, 1954b) and eicosatrienoic acid (Holman, 1956). Even if the weak-alkaline procedure gives reasonably uniform results for the di- and tri-enes, it is no more successful in this respect than the strong-alkaline method for the more unsaturated acids. Discrepancies still appear between the C_{18} and C_{20}

tetraenoic acids from sardine oil and ox adrenals respectively (TOYAMA and SHIMO-OKA, 1954b; BRICE *et al.* (1952)—compared by HERB, 1955) and between C_{20} and C_{22} pentaene acids (RIEMENSCHNEIDER, 1954).

Other anomalous findings have been reported. For example, HERB *et al.* (1951) and HERB and RIEMENSCHNEIDER 1952) isolated methyl eicosapentaenoate from cattle adrenal lipids. Later, ABU-NASR and HOLMAN (1954) prepared methyl eicosapentaenoate from cod liver oil. Under the same conditions of isomerization, the cod liver oil acid gave rise to extinction figures about 45 per cent lower than those obtained with the adrenal acid. Similarly, docosahexaenoate from cod liver oil (ABU-NASR and HOLMAN, 1954) behaved differently from the docosahexaenoate of pig brain (HAMMOND and LUNDBERG, 1953).

However, it seems doubtful if these results are necessarily indicative of a fundamental difference between fish and land animals, for TOYAMA and SHIMO-OKA (1954b) obtained an eicosapentaenoic acid from sardine oil which after alkaline isomerization gave extinction readings different from both the cattle adrenal and the cod liver oil eicosapentaenoates; on the whole it bore more resemblance to the cattle acid. As a final complication, the eicosapentaenoic acid isolated from sardine oil by WHITCUTT and SUTTON (1956) gave much lower readings than any other specimen reported. The reasons for these variations are not yet clear. They could be due, according to HERB *et al.* (1951) and HERB and RIEMENSCHNEIDER (1952), to: (a) different geometrical configurations of the acids; (b) different positions of the double bonds; or (c) the conditions of isomerization used, which although optimal for arachidonic acid, might not give the best results with the pentaenes. (This last merely restates the problem at one remove.) Unfortunately, they were unable to investigate these ideas further, owing to shortage of material. One further comment might be made: the purity of these polyunsaturated acid preparations is not always easy to assess. It is possible that mixtures might be prepared of acids with the same molecular weight and unsaturation, but having the polyallylic system in different positions. Recent work by KLENK and co-workers raises the possibility that such mixtures of polyunsaturated acids may exist in animal phosphatides (KLENK and BONGARD, 1952; KLENK and LINDLAR, 1955a,b; KLENK and DREIKE, 1955). To consider a fairly simple example: many workers call any C_{20} tetraenoic acid preparation from animal tissues 'arachidonic acid', and assume it to be eicosa-5:8:11:14-tetraenoic acid; only very rarely are degradative studies carried out. It is possible, for instance, that preparations could contain some eicosa-8:11:14:17-tetraenoic acid which might isomerize differently from arachidonic acid. [RILEY (1949) has reported that octadeca-6:9:12-trienoic acid gives extinction figures different from those of its 9:12:15-isomer, linolenic acid.] Admittedly, in the light of present knowledge it is not unreasonable to assume that C_{20} tetraene acid preparations are of eicosa-5:8:11:14-tetraenoic acid, but there is much less certainty about the penta- and hexa-ene acids.

Some light has been thrown upon the discrepancies by HAMMOND and

LUNDBERG (1953), who extended strong-alkaline isomerization to the hexaene acids by isolating and investigating methyl docosahexaenoate. They suggested that this was the hexa-cis-4:7:10:13:16-isomer, and later work of KLENK and LINDLAR (1955a) seems to provide support. HERB and RIEMENSCHNEIDER (1952) had carefully established the conditions which were optimal for arachidonic acid, and had used these for determining pentaene acids. Although HAMMOND and LUNDBERG (1953) did not make a thorough study of all the variable factors, they showed that conditions which are optimal for arachidonic are not so for the more unsaturated acids, a finding confirmed by ABU-NASR and HOLMAN (1954).

HAMMOND and LUNDBERG (1953) also found that the size of the sample used for the determination of hexaene acids was very critical, because it influenced the time taken for the sample to mix with the isomerizing solution. Any delay makes the effective heating time less than that intended and the larger the sample, the greater the difference.

It was known that delay in mixing or saponification (BRICE and SWAIN, 1945) was a cause of errors. BALDWIN and DAUBERT (1945) found their method —a slight variant of that of MITCHELL *et al.* (1943)—worked well with synthetic glycerides, and other workers have similarly found that determinations on many whole fats agree with those on the fatty acids extracted after a short saponification (e.g. on butterfat, MATTSON, 1949; MCDOWELL, 1953). But HILDITCH *et al.* (1945) had some difficulty in getting concordant linolenic-acid results on fats, a difficulty which disappeared when the fatty acids were used, suggesting that delay in saponification or mixing was responsible.

The biggest discrepancies from this cause will be seen when the extinction is changing rapidly with time, or when the time of heating is short. (That is probably why HILDITCH *et al.*, 1945, had difficulty with linolenic-containing glycerides.) In the strong-alkali determination of polyunsaturated acids, the heating time is short and the extinction developed is changing rapidly with time (Fig. 16). Any lag in mixing will greatly affect the results. A useful device for avoiding this was introduced by VANDENHEUVEL and RICHARDSON (1953): they dissolved the samples by preliminary heating at 80°. At this temperature, isomerization scarcely begins. When the sample has dissolved, the temperature is raised quickly for isomerization to start. The effective heating time will then be the same as the actual time. Using this technique, HAMMOND and LUNDBERG (1953) were able to get concordant results with methyl docosahexaenoate in samples of varying size. They suggest that some of the discrepancies found by HERB *et al.* (1951) and HERB and RIEMENSCHNEIDER (1952) may be accounted for by differences in the speed of mixing. This explanation seems unlikely to account for all the variations found by other workers—but it deserves more investigation; it may eliminate some variations found when assaying animal fats.

As sterol esters saponify only slowly, it is often impossible to determine accurately their unsaturated fatty acid content by standard alkali isomerization procedures. FRONT and DAUBERT (1945), for instance, had difficulty with

cholesteryl linoleate, which they overcame by saponifying it before isomerization. Phospholipids, too, are often hard to dissolve or saponify. The presence of sterol esters and phospholipids in fats therefore raises difficulties. Preliminary

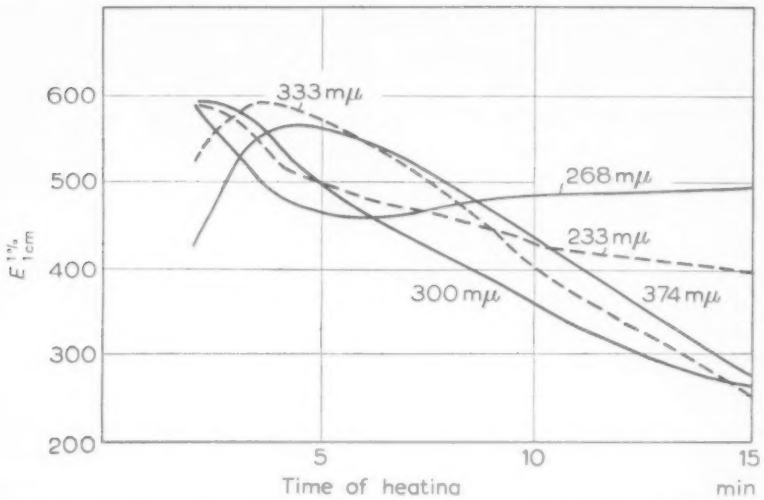

Fig. 16. Extinctions developed at various wavelengths and times during heating of docosahexaenoate in 21 per cent KOH/glycol at 180° (Hammond and Lundberg, 1953).

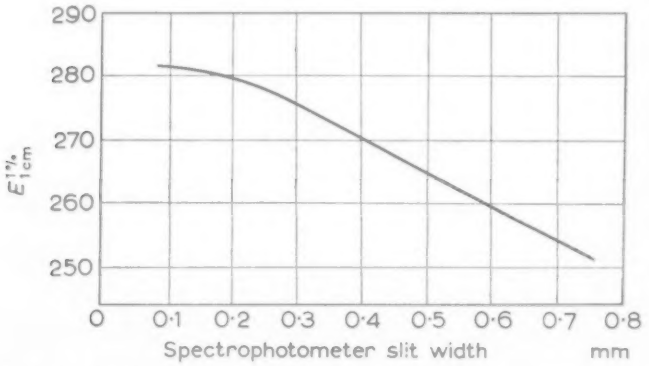

Fig. 17. Influence of spectrophotometer slit width on extinction recorded at 374 mμ for isomerized docosahexaenoate after heating in 21 per cent KOH/glycol at 180 per cent for 15 min (Hammond and Lundberg, 1953).

saponification probably cannot eliminate entirely this source of error. Even after 2 hours of saponification, O'Connell *et al.* (1952) still found apparent traces of polyunsaturated acids in the unsaponifiable fraction. Preliminary saponification for times as long as this greatly aggravates the danger of destruction of polyunsaturated acids and may cause inaccuracies as great as those which would be found if presaponification were omitted.

Hammond and Lundberg (1953) found in the slit width a further possible

source of error in determining hexaene acids. The importance of avoiding a wide slit has been mentioned earlier (see also Spectroscopy Committee, American Oil Chemists' Society, 1951), but more than routine care must be taken in the reading of the 374 mμ hexaene peak, which is abnormally sharp. Fig. 17 shows the extinction values recorded at various slit widths. Errors should be minimized by using a standard slit width, but are not completely eliminated. HERB (1955) gives details of the variation of extinction values for this 374 mμ peak, using different light sources. Given a standard slit width, however, these other discrepancies will be of negligible importance in the determination of hexaene acids where the experimental errors will almost certainly be relatively large, but they are worth bearing in mind in other spectroscopic investigations; one finds not infrequently a tendency to press results beyond the limit of instrumental accuracy.

The work of HAMMOND and LUNDBERG (1953) holds out some hope of improving determinations of penta- and hexa-ene acids, but many of the difficulties of the strong-alkali procedure remain. So far as can be seen from work published up to the time of writing, to get accurate results a good deal more concerning the nature of these polyunsaturated fatty acids and the proportions present should be known: requirements which preclude wide use of the method. Although it is thus suitable for certain limited uses, for general application to unknown fats arbitrary values have to be taken for pentaene acids, at least. In such circumstances the method can be considered as only semiquantitative. Its scope and limitations are well illustrated by HOLMAN and GREENBERG (1953). They used the procedure of HOLMAN and BURR (1948), as extended by ABU-NASR and HOLMAN (1954) to look for animal fats rich in polyunsaturated acids. They were successful in this, but one would be ill-advised to place much reliance on the analyses given. In some fats, quite substantial *negative* values for hexaenoic acid were found, larger indeed than many of the positive values reported for penta- and hexa-enoic acid contents of other fats.

It can be said with rather faint praise that the errors of strong-alkali isomerization are probably no worse than those associated with many procedures applied to polyunsaturated acids. HOLMAN and GREENBERG (1953), for instance, found that the method used to extract tissues influenced the results; variations of several hundred per cent were found in the determination of polyunsaturated acids. These were undoubtedly due in part to the shortcomings of the alkali isomerization method but probably more to the difficulty of extracting certain fatty materials, notably phospholipids, relatively rich in the polyunsaturated acids. The results they obtained, however, display clearly certain outstanding features; the high concentration of polyunsaturated acids in the male reproductive system being a notable example.

The only hope of improving the accuracy of the isomerization procedure applied to the penta- and hexa-ene acids lies in the isolation, from a variety of sources, of pure acids which could be investigated in detail, as has been done with linoleic and linolenic acids. To get such acids in sufficiently large quantities will be difficult and tedious, but worth while. At least, knowledge would be

gained of the differing behaviour of these polyunsaturated acids in varying conditions of alkali isomerization, knowledge which might be of use in distinguishing between acids or in throwing more light upon the mechanism of the reaction. At best, a much needed, more serviceable analytical method might result.

Since the work of KASS and BURR (1939), the use of potassium hydroxide and high temperatures has been general in alkaline isomerization. Choice of these conditions was made with the object of rapid and sensitive determination of linoleic acid. It is perhaps timely to consider if, for study of polyunsaturated acids, other conditions might be more serviceable. The recent preliminary report by DAVENPORT *et al.* (1956) may prove of interest. They use a molar solution of potassium tert.-butoxide in tert.-butanol, and heat at temperatures below 100°. Conjugation of linoleic and linolenic acids proceeds fairly slowly, as expected at the relatively low temperatures used, and the extinction figures are not as high as those of strong-alkaline isomerization. The interesting finding is that the conjugated chromophores appear more stable. It would be interesting to have this work extended: firstly to see to what extent naturally conjugated acids, e.g. elaeostearic, are destroyed; and secondly, to study the behaviour of the polyallylic acids. It is possible this or some similar 'low temperature' method might be more accurate with polyunsaturated acids, although it seems doubtful if it would be as sensitive as strong-alkaline isomerization.

As the conjugated products of linoleic and linolenic acid are fairly stable in these conditions, DAVENPORT *et al.* (1956) have been able to begin kinetic investigations, which might be of considerable use in predicting, more rationally than hitherto, the effects of modifications to the standard methods.

H. Artifacts

The assumption which is basic to alkali isomerization analyses is that the chromophores are produced only by migration of the ethylenic bonds of unsaturated acids. This is not wholly true. In the earlier work, e.g. BRICE *et al.* (1945), small amounts of linolenic and arachidonic acids were indicated in some oils where previous chemical investigations had given no sign of their presence. HILDITCH and SHRIVASTAVA (1947) found that several oils after alkali isomerization showed the apparent presence of linolenic acid in quantities which should have been sufficient to give a crystalline hexabromostearic acid. But this characteristic derivative could not be formed from most of the oils investigated. HILDITCH and SHRIVASTAVA concluded that small amounts of absorption around 268 mμ could not be taken as indicating linolenic acid, unless confirmed by other means. A further striking demonstration of the production of artifacts was given by BALDWIN and DAUBERT (1945). They found synthetic trilinolein after alkali isomerization showed a triene chromophore; trilinolenin similarly produced a tetraene chromophore.

SWAIN and BRICE (1949) undertook an investigation of these spurious linolenic and arachidonic acids, using vegetable oils and both freshly prepared and

autoxidized linoleic and linolenic acids. During the mild autoxidation of linolenic acid, a material forms which on alkali isomerization gives the same tetraene chromophore as does arachidonic acid. This oxidation product can be distinguished from arachidonic acid, as it conjugates on heating in *neutral* ethylene glycol; arachidonic will do this only in alkali. The amount of tetraene absorption formed in many oils by heating alone, i.e. due not to arachidonic acid but to an oxidation artifact, was equal to or greater than that formed on alkali isomerization. This would previously have been reported as arachidonic acid, whereas it is manifestly an oxidation product of linolenic acid. Similarly, autoxidized linoleic acid on alkali isomerization gave a triene chromophore apparently the same as that of linolenic acid; but this was much less marked.

Autoxidized fatty acids show before isomerization a peak near 234 mμ as do—to take examples investigated by SWAIN and BRICE (1949)—some vegetable oils and, also, beef fat. These preformed diene peaks behaved differently: in beef fat they were stable to heating (as are the known conjugated linoleic acids); in most vegetable oils, and, more markedly, in autoxidized fatty acids, they fell. SWAIN and BRICE (1949) therefore deduced that conjugated linoleic acid was responsible for the peak in beef fat; in autoxidized fatty acids, and to a certain extent in oils, there must be some other, less stable chromophore.

PRIVETT and LUNDBERG (1951) investigated further the changes produced by autoxidation of methyl linoleate and linolenate on the spectral characteristics following alkali isomerization. The figures they give are of interest in indicating the sort and size of errors that may arise from oxidation products. For example, autoxidized linolenate (peroxide value: 2300 m.e./kg) analysed as 66·9 per cent linolenic, 17·8 per cent arachidonic, 15·7 per cent conjugated diene and 0·7 per cent conjugated triene.

Most of this work was done on fatty acids oxidized far beyond what usually occurs in natural fats. In normal alkali isomerizations, the errors introduced by these interfering substances are not great. Yet they do exist and are recognized to be there; there is general agreement to ignore (except as an index of the state of oxidation) any small tetra- or tri-ene conjugation found in an oil which should not contain any linolenic or arachidonic acid. The conclusion seems inescapable that in fats which truly contain linolenic or arachidonic acid a part of that found after alkali isomerization may well be an artifact.

SWAIN and BRICE (1949) suggested that these artifact chromogens could be determined by heating a sample of the oil in neutral glycol; figures obtained in alkali isomerization could then be corrected. But this is not a quantitative procedure. For example, SWAIN and BRICE found that autoxidized linoleic acid on alkali isomerization appeared to contain 0·26 per cent of linolenic acid. If instead, it was heated in neutral glycol, it appeared to have 8·2 per cent linolenic acid. It is obvious that use of this 'correction' device can introduce larger errors than would be caused by accepting alkali isomerization data at their face value.

A better procedure was devised by PRIVETT and LUNDBERG (1951): the oxidized and unoxidized portions of autoxidized fatty acid esters were separated

by countercurrent distribution. By this means it was possible to analyse fats that had undergone some oxidation. This method is too time-consuming to apply to routine fat determinations and there is no satisfactory way of correcting for small amounts of interfering oxidation products. Where fats have undergone even a small amount of oxidation, spectroscopic results must be interpreted with caution.

I. Adaptation to semi-micro and micro scale

The analytical alkali isomerization procedures in current use need only small amounts of fat (about 100 mg) but even these are prohibitively large for many biological investigations. Variations of the standard procedure have been tried using much smaller samples (see KRETCHMER, 1948).

BERK *et al.* (1950) devised a method using 100 μg of fat. With glycol or glycerol as the isomerization solvent, they found the blanks to be too high and too variable, and, as the solutions did not have to be diluted much, glycol and glycerol were too viscous to use as isomerization solvents. These workers therefore made use of the discovery of BRADLEY and RICHARDSON (1942) that alkali-isomerization will occur in water—if the temperature is high enough. BERK *et al.* heated an aqueous alkaline solution in a bomb by passing in steam under pressure. With such very small samples, relatively great destruction can be caused by traces of oxidizing substances; reproducibility was improved by adding small amounts of hydroquinone as antioxidant. This useful precaution has been used by WIESE and HANSEN (1953) in devising their own procedure (*vide infra*). In its published form, however, the method of BERK *et al.* (1950) seems unlikely to have wider use. The empirical nature of the procedure is well brought out by their comment that new constants had to be determined whenever a repair or adjustment was made to the bomb.

WIESE and HANSEN (1953) have developed a method for the determination of di-, tri-, and tetra-enoic acids in 3 ml of plasma. In its present form this gives high readings for tetraenoic acids because pentaene acids interfere (WIESE, GIBBS, and HANSEN, 1954). WIESE *et al.* (1954) were able to extend the method to include hexaenoic acids, but had no suitable pentaene standard of reference. Pentaenoic acids therefore cannot be determined by this method, although they are known to exist in plasma (HAMMOND and LUNDBERG, 1955; EVANS, WALDRON, OLEKSYSHYN, and RIEMENSCHNEIDER, 1956).

The higher extinctions obtainable by the use of strong alkali have obvious possibilities for a micro method; HOLMAN and BURR (1948) were able to use samples smaller than those taken by previous workers. The greater need, however, was for methods of determining the polyunsaturated acids and here progress had to wait upon the availability of suitable standards. Once these were available, HERB and RIEMENSCHNEIDER (1953) were able to adapt their strong-alkali method to fat samples of 1–10 mg. At the time of writing, it is the most widely used micro method and has been employed successfully for investigations of differences in a given organ or tissue associated with a specific dietary or other change, e.g. KLEIN and JOHNSON (1954); EVANS, RIEMEN-

SCHNEIDER and HERB (1954). It suffers, of course, from the limitations which must be accepted on any strong-alkali procedure applied to polyunsaturated acids; for example, the results of HAMMOND and LUNDBERG (1955) and EVANS *et al.* (1956) on the unsaturated acids of human plasma differ somewhat. A further modification of this micro procedure of HERB and RIEMENSCHNEIDER has been developed by HOLMAN (1956).

J. Standard methods

Experience with alkali isomerization shows that the experimental conditions must be adhered to within very narrow limits if reproducible results are to be obtained. If the conditions are defined precisely, conversion factors determined for pure acids can be used by other workers (and are very valuable for the more uncommon acids which are not readily obtainable in a pure state). The first of these carefully specified procedures was that of MITCHELL *et al.* (1943) (p. 246). Greater knowledge of the variables has led to some revision of the original method. In the currently accepted form of the weak-alkali determination of linoleic and linolenic acids a 200-mg sample of fat is first examined spectroscopically for conjugated constituents. A one-inch-diameter test tube containing 11 g of a 6·5–6·6 per cent (w/w) solution of potassium hydroxide in glycol is immersed to a depth of $4\frac{1}{2}$ in. in a bath at 180°C $\pm$ 0·5°C. The glycol is protected from atmospheric oxidation by a current of nitrogen (containing not more than 0·01 per cent oxygen) flowing at a minimum rate of 50 ml per min. When the glycol has reached 180°C, a 100-mg sample of fat is dropped in. The tube is withdrawn from the bath, swirled vigorously for a few seconds to mix the contents, and returned to the bath. At the end of one minute's heating, if the contents are not clear, indicating that solution or saponification is incomplete, the tube is again swirled two or three times. Exactly 25 min after the fat sample is added, the tube is removed from the bath and cooled. The contents are then diluted and the extinction read at 233 mμ, 262 mμ, 268 mμ, and 274 mμ. Correction procedures are applied if necessary, and from the corrected figures obtained the contents of linoleic and linolenic acids are calculated using factors based on data obtained with pure all-cis-acids (see Table 3).

Fuller experimental details are given by the Spectroscopy Committee, American Oil Chemists' Society (1949; 1951), and various workers have suggested other minor improvements, e.g. MORETTI and CHEFTEL (1955); SAKURAGI and KUMMEROW (1956), but it is obvious that the procedure is still essentially the same as that devised by MITCHELL *et al.* (1943).

As a result, however, of the intense study of the method described in this review, other standard procedures have been developed. Probably the most useful of these are the determination of unsaturated acids up to hexaenes by heating for 15 min in 21 per cent potassium hydroxide (HERB and RIEMENSCHNEIDER, 1952) and its micro modification (HERB and RIEMENSCHNEIDER, 1953). These strong-alkali isomerization methods produce more intense absorption from the polyunsaturated acids (see Table 3).

Table 3. Extinctions produced from polyunsaturated acids by standard alkali-isomerization procedures

Acid	λ (mμ)	$E^{1\text{ per cent}}_{1\text{ cm}}$	
		6·5% KOH: 25 min	21% KOH: 15 min
Linoleic	233	921	916
Linolenic	233	616	475
	268	507	905
Arachidonic	233	569	397
	268	528	482
	315	215	606
Pentaenoic	233		415
(50% C_{20}; 50% C_{22})	268		436
	315		697
	346		690
Hexaenoic	233		417
	268		522
	315		296
	346		277
	375		293

Data of HERB and RIEMENSCHNEIDER (1952, 1953) and RIEMENSCHNEIDER (1954).

K. Efficacy of the method

Although the full working instructions for weak-alkali isomerization (Spectroscopy Committee, American Oil Chemists' Society, 1949, 1951) specify many precautions which must be taken to obtain reproducible results, even those are considered inadequate by VANDENHEUVEL and RICHARDSON (1953). They describe a modified procedure which, they claim, gives improved accuracy and reliability. However, they cite results only on pure specimens of fatty acids; it would be interesting to have their procedure independently compared with the standard method of the American Oil Chemists' Society. Analysts skilled in the latter procedure have little need to improve reproducibility: collaborative surveys organized by the above Spectroscopy Committee (1949, 1951, 1953, 1955) show that good agreement can be reached between different laboratories. Nevertheless, occasional deviations of up to 10 per cent are found, and in the 1951 tests one of the six collaborating laboratories was eliminated from the survey because of grossly inconsistent results. Perhaps the good agreement between the other collaborators was due more to skill at the bench than to the virtues of the method itself. VANDENHEUVEL and RICHARDSON (1953) roundly describe the alkali-isomerization procedure as "a long, complicated, exacting series of manipulations requiring above average skill and experience." Few would disagree with this judgement.

Two distinct tendencies are apparent when the development of the method is surveyed. Some workers have tried at all costs to improve its precision and accuracy. This commendable drive for perfection has resulted in a procedure too elaborate for those who do not carry out alkali isomerizations routinely.

Such workers may resist modifications which improve accuracy on the grounds that the improvement attainable does not justify the extra work or the acquisition of a special skill. This attitude accounts for the continued (and widespread) use of older procedures which are known to be slightly inaccurate.

Nevertheless, by taking great care, the method can be made precise. Is it equally accurate? No ready answer can be given. Results obtained by spectrophotometric means can at present be checked only against values obtained from iodine and thiocyanogen numbers, which themselves depend on empirical factors. Agreement is fairly good for cottonseed and soya-bean oils, which contain di- and tri-enoic acids (Spectroscopy Committee, American Oil Chemists' Society, 1951; O'Connor *et al.*, 1952). Iodine and thiocyanogen values give slightly higher figures for dienoic acids and distinctly higher for tri-enoic acids than does the spectroscopic method. That does not necessarily imply that the alkali isomerization procedure is inaccurate; the fault may well be with the iodine-thiocyanogen values. It is probably true that for oils containing substantial amounts of di- and tri-enoic acids, but no polyunsaturated acids, the spectrophotometric method is as accurate as any. It is now entrenched as the best routine method, and indeed a nomograph has been published enabling rapid calculation from extinction coefficients and iodine values of the fatty acid composition of samples (Narayan and Kulkarni, 1954).

When smaller amounts of unsaturated acids are present, results may be less reliable partly because of artifacts and partly because of difficulties in correcting for irrelevant absorption. Here again, however, the inaccuracies are probably less than those attending the use of any available alternative method.

It is in respect of the analysis of fats containing tetraenoic and higher unsaturated acids that the method is least satisfactory. Variations in behaviour are apparent between unsaturated acids of different chain lengths and even between acids of the same chain length isolated from different sources. These anomalies seem to preclude the development of a general method capable of giving absolute results on any and every fat. Semiquantitative estimations of small amounts of polyunsaturated acids can be made (no other method will do as much) and similar fats can be compared for their content of such acids. These are limited but very useful achievements.

It is disappointing that the alkali isomerization method must be circumscribed, in spite of the great efforts that have been made to devise general procedures. Unfortunately, it is now apparent that the "general" methods developed are not capable of unlimited application. A procedure very suitable for one fat may be useless for another. Methods perfected for natural vegetable oils containing linoleic and linolenic acids give false results for fish oils or for oils which have undergone oxidation or have been processed in certain ways. Even with natural vegetable oils great care is often necessary in interpreting the results. It is common to discount small amounts of tri- and tetra-enoic acids on the grounds that they should not be there; this may seem hazardous and arbitrary but it is a lesser evil. Given a certain wariness, however, the method may be used widely and fairly safely.

Reiterating, alkali isomerization is an empirical method; the only justification it needs is that it should give serviceable results. By this criterion it has been very successful and there is no substitute for it. Admittedly it has been shown to have serious limitations but the researches which have revealed them have lacked neither insight nor interest.

We wish to thank Drs. F. D. Gunstone, J. McLean, E. M. Meade, N. Polgar, J. P. Riley, B. C. L. Weedon, and Dr. L. A. Jordan, Dr. S. H. Bell, Mr. N. H. E. Ahlers and the Research Association of British Paint, Colour, and Varnish Manufacturers for providing unpublished information. We are also grateful to the authors and the editors of the following journals for permission to reproduce figures: *Journal of the Chemical Society* (Fig. 2), *Journal of the American Oil Chemists' Society* (Figs. 4, 9, 12–17), *Analytical Chemistry* (Fig. 7), *Journal of the Optical Society of America* (Figs. 8 and 11), *Analyst* (Fig. 9).

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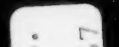

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